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**APPLICATION FOR UNITED STATES LETTERS PATENT**  
**FOR**  
**ISOLATION OF BINDING PROTEINS WITH HIGH AFFINITY TO LIGANDS**

by

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## BACKGROUND OF THE INVENTION

The government owns rights in the invention pursuant to DARPA Grant No. MDA(972-97-0009).

### **1. Field of the Invention**

The present invention relates generally to the field of protein engineering. More particularly, it concerns methods for the screening of combinatorial libraries of polypeptides to allow isolation of enzymes having a desired catalytic activity and of ligand binding proteins, including antibodies and binding proteins having affinity for selected ligands

### **2. Description of Related Art**

The isolation of proteins that either bind to ligands with high affinity and specificity or catalyze the enzymatic conversion of a reactant (substrate) into a desired product is a key process in biotechnology. Ligand-binding proteins and enzymes with a desired substrate specificity can be isolated from large libraries of mutants, provided that a suitable screening method is available. Small protein libraries composed of  $10^3$ - $10^5$  distinct mutants can be screened by first growing each clone separately and then using a conventional assay for detecting clones that exhibit specific binding. For example, individual clones expressing different protein mutants can be grown in microtiter well plates or separate colonies on semisolid media such as agar plates. To detect binding the cells are lysed to release the proteins and the lysates are transferred to nylon filters, which are then probed using radiolabeled or fluorescently labeled ligands (DeWildt *et al.* 2000). However, even with robotic automation and digital image systems for detecting binding in high density arrays, it is not feasible to screen large libraries consisting of tens of millions or billions of clones. The screening of libraries of that size is required for the *de novo* isolation of enzymes or protein binders that have affinities in the nanomolar range.

The screening of very large protein libraries has been accomplished by a variety of techniques that rely on the display of proteins on the surface of viruses or cells (Ladner *et al.*

1993). The underlying premise of display technologies is that proteins engineered to be anchored on the external surface of biological particles (*i.e.*, cells or viruses) are directly accessible for binding to ligands without the need for lysing the cells. Viruses or cells displaying proteins with affinity for a ligand can be isolated in a variety of ways including sequential adsorption/desorption from immobilized ligand, by magnetic separations or by flow cytometry (Ladner *et al.* 1993, U.S. Patent 5,223,409, Ladner *et al.* 1998, US patent 5,837,500, Georgiou *et al.* 1997, Shusta *et al.* 1999). The most widely used display technology for protein library screening applications is phage display. Phage display is a well-established and powerful technique for the discovery of proteins that bind to specific ligands and for the engineering of binding affinity and specificity (Rodi and Makowski, 1999). In phage display, a gene of interest is fused in-frame to phage genes encoding surface-exposed proteins, most commonly pIII. The gene fusions are translated into chimeric proteins in which the two domains fold independently. Phage displaying a protein with binding affinity for a ligand can be readily enriched by selective adsorption onto immobilized ligand, a process known as "panning". The bound phage is desorbed from the surface, usually by acid elution, and amplified through infection of *E. coli* cells. Usually, 4-6 rounds of panning and amplification are sufficient to select for phage displaying specific polypeptides, even from very large libraries with diversities up to  $10^{10}$ . Several variations of phage display for the rapid enrichment of clones displaying tightly binding polypeptides have been developed (Duenas and Borrebaeck, 1994; Malmborg *et al.*, 1996; Kjaer *et al.*, 1998; Burioni *et al.*, 1998; Levitan, 1998; Mutuberria *et al.*, 1999; Johns *et al.*, 2000).

One of the most significant applications of phage display technology has been the isolation of high affinity antibodies (Dall'Acqua and Carter, 1998; Hudson *et al.*, 1998; Hoogenboom *et al.*, 1998; Maynard and Georgiou, 2000). Very large and structurally diverse libraries of scFv or F<sub>AB</sub> fragments have been constructed and have been used successfully for the *in vitro* isolation of antibodies to a multitude of both synthetic and natural antigens (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; Sheets *et al.*, 1998; Pini *et al.*, 1998; de Haard *et al.*, 1999; Knappik *et al.*, 2000; Sblattero and Bradbury, 2000). Antibody fragments with improved affinity or specificity can be isolated from libraries in which a chosen antibody had been subjected to mutagenesis of either the CDRs or of the entire gene

CDRs (Hawkins *et al.*, 1992; Low *et al.*, 1996; Thompson *et al.*, 1996; Chowdhury and Pastan, 1999). Finally, the expression characteristics of scFv, notorious for their poor solubility, have also been improved by phage display of mutant libraries (Deng *et al.*, 1994; Coia *et al.*, 1997).

However, several spectacular successes notwithstanding, the screening of phage-displayed libraries can be complicated by a number of factors. First, phage display imposes minimal selection for proper expression in bacteria by virtue of the low expression levels of antibody fragment gene III fusion necessary to allow phage assembly and yet sustain cell growth (Krebber *et al.*, 1996, 1997). As a result, the clones isolated after several rounds of panning are frequently difficult to produce on a preparative scale in *E. coli*. Second, although phage displayed proteins may bind a ligand, in some cases their un-fused soluble counterparts may not (Griep *et al.*, 1999). Third, the isolation of ligand-binding proteins and more specifically antibodies having high binding affinities can be complicated by avidity effects by virtue of the need for gene III protein to be present at around 5 copies per virion to complete phage assembly. Even with systems that result in predominantly monovalent protein display, there is nearly always a small fraction of clones that contain multiple copies of the protein. Such clones bind to the immobilized surface more tightly and are enriched relative to monovalent phage with higher affinities (Deng *et al.*, 1995; MacKenzie *et al.*, 1996, 1998). Fourth, theoretical analysis aside (Levitan, 1998), panning is still a “black box” process in that the effects of experimental conditions, for example the stringency of washing steps to remove weakly or non-specifically bound phage, can only be determined by trial and error based on the final outcome of the experiment. Finally, even though pIII and to a lesser extent the other proteins of the phage coat are generally tolerant to the fusion of heterologous polypeptides, the need to be incorporated into the phage biogenesis process imposes biological constraints that can limit library diversity. Therefore, there is a great need in the art for techniques capable of overcoming these limitations.

Protein libraries have also been displayed on the surface of bacteria, fungi, or higher cells. Cell displayed libraries are typically screened by flow cytometry (Georgiou *et al.* 1997, Daugherty *et al.* 2000). However, just as in phage display, the protein has to be

engineered for expression on the cell surface. This imposes several potential limitations. First of all, either the N-terminal or the C-terminal of the protein has to be fused to a vehicle for display. Thus, these technologies are not suitable where the N- or C- termini are essential for ligand binding. Second, the requirement for display of the protein on the surface of a cell imposes biological constraints that limit the diversity of the proteins and protein mutants that can be screened. Third, complex proteins consisting of several polypeptide chains cannot be readily displayed on the surface of bacteria, filamentous phages or yeast. As such, there is a great need in the art for technology which circumvents all the above limitations and provides an entirely novel means for the screening of very large polypeptide libraries.

At present, the isolation of novel enzymes from libraries of protein mutants is typically accomplished either through the use of a phenotypic selection or screening in either solid phase or microtiter well plates. Biological selections are based on complementation of auxotrophy or resistance to cytotoxic agents (*e.g.*, antibiotics). Unfortunately, the utility of phenotypic selections is limited to the isolation of catalysts for reactions that are of direct biological relevance or can be indirectly linked to a selectable phenotype. Alternatively, each clone in a mutant population may be screened directly for enzymatic activity. For libraries expressed in microorganisms, screening can be performed on colonies growing on a solid substrate such as agar. Solid phase screening relies on substrates of an enzymatic reaction that give rise to a zone of clearance, a fluorescent product, or a strongly absorbing (chromogenic) product. The assay may detect the enzyme product directly or may be coupled to a second enzyme whose product can in turn be easily monitored. However, many assays cannot be implemented in a solid phase format. If that is the case then individual clones must be grown and assayed in microtiter wells. Such assays are significantly more time consuming than solid phase assays and severely limit the number of mutants that can be screened. However, when a small number of random mutants is screened, the probability of finding clones expressing an enzyme that can catalyze a desired biotransformation, especially when that biotransformation requires a complicated reaction, is severely affected.

In general, methods that will allow the screening of large libraries of enzyme mutants on the basis of kinetic parameters, *i.e.*, on the basis of how much product is generated per

unit time, are needed. Phage display technology may in principle be used as a tool for the isolation of useful enzymes from large libraries. However, harnessing phage display technology for the isolation of enzyme catalysts from libraries has thus far not proven practical (Olsen et al. 2000). For example there is no apparent way to physically link in a quantitative manner a phage particle displaying a certain enzyme clone with the outcome of multiple catalytic turnovers resulting in the accumulation of reaction product. Establishing such a linkage is essential for the screening of protein libraries on the basis of catalytic proficiency.

### **SUMMARY OF THE INVENTION**

In one aspect, the invention provides a method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand, the method comprising the steps of: (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein the binding protein is expressed in soluble form in the bacterium; (b) contacting the bacterium with a labeled ligand capable of diffusing into the bacterium; and (c) selecting the bacterium based on the presence of the labeled ligand within the bacterium, wherein the ligand and the candidate binding protein are bound in the bacterium.

In another aspect, the invention provides a method of obtaining a nucleic acid sequence encoding a binding protein capable of binding a target ligand, the method comprising the step of: (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein the binding protein is expressed in soluble form in the bacterium; (b) contacting the bacterium with a labeled ligand capable of diffusing into the bacterium; (c) selecting the bacterium based on the presence of the labeled ligand within the bacterium, wherein the ligand and the candidate binding protein are bound in the bacterium; and (d) cloning the nucleic acid sequence encoding the candidate binding protein.

In another aspect of the invention, the binding protein expressed in the bacterium is further defined as expressed in soluble form in the periplasm of the bacterium. The nucleic acid sequence encoding the binding protein may still further be defined as encoding a nucleic acid sequence comprising the candidate binding protein sequence operably linked to a leader sequence capable of directing expression of the candidate binding protein in the periplasm. Potentially any Gram negative bacterium could be used with the invention, including, for example, an *E. coli* bacterium. In one embodiment of the invention, the nucleic acid sequence encoding a candidate binding protein may be further defined as capable of being amplified following the selection. The invention may still further be defined as including removing labeled ligand not bound to the candidate binding protein.

In yet another aspect, the invention comprises providing a population of Gram negative bacteria. In one embodiment of the invention, the population of bacteria is further defined as collectively capable of expressing a plurality of candidate binding proteins. In yet another embodiment of the invention, the population of bacteria is obtained by a method comprising the steps of: a) preparing a plurality DNA inserts which collectively encode a plurality of different potential binding proteins, and b) transforming a population of gram negative bacteria with the DNA inserts. The population of Gram negative bacteria may be still further defined as contacted with the labeled ligand.

In still yet another aspect of the invention, a candidate binding protein employed in accordance with the invention is further defined as an antibody or fragment thereof, or alternatively, is a binding protein other than an antibody. Still further, the candidate binding protein may be an enzyme, including any portion thereof. A candidate binding protein used with the invention may be further defined as not capable of diffusing out of the periplasm in intact bacteria.

In still yet another aspect of the invention, a labeled ligand may comprise a polypeptide, an enzyme and / or a nucleic acid or the like. The labeled ligand may be further defined as comprising a molecular weight of less than about 20,000 Da, less than about 10,000 Da or less than about 5,000 Da, and may in other embodiments of the invention be

described as greater than 600 Da in molecular weight. The labeled ligand may be still further defined as fluorescently labeled.

In still yet another aspect, the invention comprises treating a bacterium to facilitate diffusing of a target ligand into the periplasm. In certain embodiments of the invention, the treating may comprise use of hyperosmotic conditions, physical stress, treating the bacterium with a phage, or growing the bacterium at a sub-physiological temperature, for example, about 25°C.

In still yet another aspect, the invention comprises selecting one or more bacteria using FACS or magnetic separation. In the invention, the ligand and candidate binding protein may be further defined as reversibly bound in the periplasm.

In still yet another aspect, the invention provides a method of obtaining a bacterium comprising a nucleic acid sequence encoding a catalytic protein catalyzing a chemical reaction involving a target substrate, the method comprising the steps of: (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate catalytic protein, wherein the catalytic protein is expressed in soluble form in the bacterium; (b) contacting the bacterium with a target substrate capable of diffusing into the bacterium, wherein the candidate catalytic protein catalyzes a chemical reaction involving the target substrate and wherein the chemical reaction yields at least a first substrate product; and (c) selecting the bacterium based on the presence of the first substrate product. In yet another aspect of the invention, the method may be further defined as a method of obtaining a nucleic acid sequence encoding a catalytic protein catalyzing a reaction with a target substrate, the method further comprising the step of: (d) cloning the nucleic acid sequence encoding the candidate catalytic protein. By "catalytic protein" it is meant a molecule which is capable of increasing the rate of a chemical reaction relative to the rate the reaction would occur absent the catalytic protein. In the method, the candidate catalytic protein may be expressed in soluble form in the periplasm of the bacterium. The nucleic acid sequence encoding a candidate catalytic protein may, in further embodiments of the invention, be defined as

operably linked to a leader sequence capable of directing expression of the candidate catalytic protein in the periplasm.

In still yet another aspect, the aforementioned method may be carried out with any Gram negative bacterium, for example, an *E. coli* bacterium. The invention may also comprise providing a population of Gram negative bacteria. The population may be further defined as collectively capable of expressing a plurality of candidate catalytic proteins. In one embodiment of the invention, the population of bacteria is obtained by a method comprising the steps of: a) preparing a plurality DNA inserts which collectively encode a plurality of different candidate catalytic proteins, and b) transforming a population of Gram negative bacteria with the DNA inserts. The Gram negative bacteria may be defined as contacted with the target substrate. A bacterium selected with the invention may be further defined as viable following the selecting. Selecting may be carried out by any desired method, for example, FACS or magnetic separation.

In still yet another aspect of the invention, a candidate catalytic protein is an enzyme. The candidate catalytic protein may also be defined as not capable of diffusing out of the periplasm.

In still yet another aspect of the invention, a target substrate may comprise a molecule containing a scissile amide bond. The target substrate may also comprise a polypeptide or a nucleic acid. In certain embodiments of the invention, the target substrate comprises a molecule containing a scissile carboxylic ester bond, a molecule containing a scissile phosphate ester bond, a molecule containing a scissile sulfonate ester bond, a molecule containing a scissile carbonate ester bond, a molecule containing a scissile carbamate bond, and / or a molecule containing a scissile thioester bond. In still further embodiments of the invention, the target substrate is further defined as comprising a molecular weight of less than about 20,000 Da, less than about 5,000 Da, less than about 3,000 Da, or may be defined as comprising a molecular weight of greater than about 600 Da, including from about 600 Da to about 30,000 Da.

In still yet another aspect of the invention, the first substrate product is further defined as capable of being detected based on the presence of a fluorescent signature. This fluorescent signature may be absent in the target substrate, and may only be produced upon chemical reaction to produce the first substrate product. In one embodiment of the invention, a fluorescent signature is produced by catalytic cleavage of a scissile bond. The method may be further defined as comprising use of a FRET system, the FRET system comprising a fluorophore bound by a scissile bond to at least a first molecule capable of quenching the fluorescence of the fluorophore, wherein cleavage of the scissile bond allows the first molecule to diffuse away from the fluorophore and wherein the fluorescence of the fluorophore becomes detectable. In the method, the fluorophore may comprise a positive charge allowing the fluorophore to remain associated with the bacterium. In further embodiments of the invention, the target substrate may be further defined as comprising a latent fluorescent moiety capable of being released by the chemical reaction involving the target substrate. The latent fluorescent moiety released by the cleavage may possess an overall positive charge allowing the moiety to remain associated with the bacterium following the cleavage. In yet another embodiment of the invention, the method may comprise labeling the target substrate with a fluorescent pH probe capable of being detected upon a change in pH associated with the chemical reaction involving the target substrate. The fluorescent pH probe may possess an overall positive charge allowing the fluorescent pH probe to remain associated with the bacterium following the chemical reaction involving the target substrate. It will be understood by those of skill in the art that multiple products may be produced as a result of the chemical reaction and any one or more of these could potentially be detected to reveal that occurrence of the chemical reaction. Accordingly, the reaction product may comprise a change in pH within the bacterium which could be detected.

In still yet another aspect, the invention may comprise treating the bacterium to facilitate the diffusing into the periplasm, for example, using hyperosmotic conditions, physical stress, treating the bacterium with a phage, and growing the bacterium at a sub-physiological temperature, for example, about 25°C.

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**FIG. 2.** Isolation of affinity improved mutants of an anti-digoxin antibody by two rounds of sorting. A library of scFv mutants in which three residues in the light chain had been randomized was constructed as described in Example 2. A total of  $2.5 \times 10^6$  transformants were grown in liquid media, labeled with 100 nM digoxin-BODIPY™ and fluorescent cells falling within the window shown in the rightmost panel were sorted by FACS. The sorted cells were grown in liquid media, re-labeled and cells falling within the specified window as shown in the center panel were isolated. Following a final round of re-growth the cells were analyzed by FACS (left-most panel). Single scFv antibody colonies were picked at random, analyzed and the affinity of the corresponding scFv proteins are reported in Table 1.

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**FIG. 4.** Effect of hyperosmotic shock on labeling efficiency: (i)–(iv), pHEN2.thy; (v)–(viii), pHEN2.dig; (i) and (v), 1xPBS; (ii) and (vi), 2.5xPBS; (iii) and (vii) 5xPBS; (iv) and (viii) 10xPBS.

**FIG. 5.** Maximizing periplasmic FACS signal in ABLE<sup>TM</sup>C labeled in 5xPBS using P<sub>tac</sub> vector and superinfection with M13KO7 (moi of 10) 0.5h pre-induction: i) pHEN.thy; ii) pHEN2.thy/M13K07; pHEN2.dig; iv) pHEN2.dig/M13K07.

**FIG. 6A-C.** FIG 6A: Phage eluate titers, after each round of panning. FIG. 6B: Polyclonal phage ELISA of purified phage stocks on digoxin-ovalbumin. FIG. 6C: FACSScanning naïve library (i) and rounds one to five (ii to vi) of panning on digoxin-BSA using BODIPY<sup>TM</sup>-digoxigenin.

**FIG. 7A, B.** Amino acid and nucleotide sequences of scFv antibody fragments isolated by expression in the periplasm and FACS. FIG. 7A: Heavy chain of dig1 is shown in true font while dig3 is shown in italics underneath. The nucleotide sequences corresponding to the heavy chains of dig1 and dig 3 are given by SEQ ID NO:17 and SEQ ID NO:18, respectively. Dig2 variation from dig 1 is as indicated in underlined text within CDR3. FIG. 7B: Light chain of dig1, 2 and 3 with variations in CDR3 indicated as for heavy chain. The nucleotide sequences corresponding to the light chains of dig1 and dig 3 are given by SEQ ID NO:19 and SEQ ID NO:20, respectively. The underlined four nucleotide variation beginning at nucleotide 99 is given by SEQ ID NO: 21.

**FIG 8.** Labeling of periplasmic scFv by fluorescently tagged oligonucleotide probe. ABLE<sup>TM</sup>C cells expressing periplasmic scFv specific for either atrazine as a negative control (i and iii) or for digoxin (ii and iv) were labeled either with: 100nM with digoxigenin-BODIPY<sup>TM</sup> (i and ii) or 100nM of dig-5A-FL (iii and iv). 10,000 events were recorded using a FACSort flow cytometer at a rate of approximately 1,000 events per second.

**FIG 9.** Fluorescence discrimination of *E. coli* expressing the enzyme cutinase (an esterase) from control bacteria not expressing the enzyme. *E. coli* DH5a cells were

## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In wild-type *E. coli* and other gram negative bacteria, the outer membrane serves as a permeability barrier that severely restricts the diffusion of molecules greater than 600 Da into the periplasmic space (Decad and Nikado, 1976). Conditions that increase the permeability of the outer membrane, allowing larger molecules to diffuse in the periplasm, have two deleterious effects in terms of the ability to screen libraries: (a) the cell viability is affected to a significant degree and (b) the diffusion of molecules into the cell is accompanied by the diffusion of proteins and other macromolecules. The expressed proteins leak out of the periplasm and thus the resulting cell based libraries cannot be screened in a meaningful way.

However, the inventors have identified experimental conditions such that allow fluorescent conjugates of ligands and polypeptides equilibrate across the outer membrane while proteins secreted into the periplasm remain within the cell. Therefore, in bacterial cells expressing recombinant polypeptides with affinity for the ligand, the concentration of the labeled ligand bound to the binding protein is increased, allowing the bacteria to be isolated from the rest of the library. Where fluorescent labeling of the target ligand is used, cells may efficiently be isolated by fluorescence activated cell sorting (FACS). Thus, in effect, the cell envelope serves as a “dialysis bag” that retains macromolecule:ligand complexes, but not free ligand (FIG. 1). With this approach, existing libraries of secreted proteins in bacteria can be easily tested for ligand binding without the need for subcloning into a phage or cell surface display system.

#### **I. Anchor-less Display Library Screening**

Prior art methods of both phage display and bacterial cell surface display suffer from a limitation in that the protein is required, by definition, to be physically displayed on the surface of the vehicle used, to allow unlimited access to the targets (immobilized for phage or fluorescently conjugated ligands for FACS) (U.S. Patent 5,223,409, the disclosure of which is specifically incorporated herein by reference in its entirety). Certain proteins are known to be poorly displayed on phage (Maenaka *et al.*, 1996; Corey *et al.*, 1993) and the toxic effects of cell surface display have been treated at length (Daugherty *et al.*, 1999). The proteins to be displayed also need to be expressed as fusion proteins, which may alter their function. The selection constraints imposed by any display system may, therefore, limit the application to relatively small and “simple” proteins and deny access to a multitude of large and complex multisubunit species. The latter are very likely to be incapable of partaking efficiently in the complex process of phage assembly termination or outer-membrane translocation without very serious effects on host cell viability.

Herein, the inventors have described conditions whereby expressed binding proteins, for example, an antibody, are targeted to the periplasmic compartment of *E. coli* and yet are amenable to binding ligands and peptides of up to at least 2kDa. As used herein, the term “binding protein” includes not only antibodies, but also fragments of antibodies, as well as

any other polypeptide or protein potentially capable of binding a given target molecule. The antibody or other binding proteins may be expressed with the invention directly and not as fusion proteins. Such a technique may be termed "anchor-less-display" (ALD). To understand how it may work, one needs to be aware of the location in which it functions.

The periplasmic compartment is contained between the inner and outer membranes of Gram negative cells (see, *e.g.*, Oliver, 1996). As a sub-cellular compartment, it is subject to variations in size, shape and content that accompany the growth and division of the cell. Within a framework of peptidoglycan heteropolymer is a dense milieu of periplasmic proteins and little water, lending a gel-like consistency to the compartment (Hobot *et al.*, 1984; van Wielink and Duine, 1990). The peptidoglycan is polymerized to different extents depending on the proximity to the outer membrane, close-up it forms the murein sacculus that affords cell shape and resistance to osmotic lysis.

The outer membrane (see Nikaido, 1996) is composed of phospholipids, porin proteins and, extending into the medium, lipopolysaccharide (LPS). The molecular basis of outer membrane integrity resides with LPS ability to bind divalent cations ( $Mg^{2+}$  and  $Ca^{2+}$ ) and link each other electrostatically to form a highly ordered quasi-crystalline ordered "tiled roof" on the surface (Labischinski *et al.*, 1985). The membrane forms a very strict permeability barrier of allowing passage of molecules no greater than around 650 Da (Burman *et al.*, 1972; Decad and Nikaido, 1976) via the porins. The large water filled porin channels are primarily responsible for allowing free passage of mono and disaccharides, ions and amino acids in to the periplasm compartment (Naeke, 1976; Nikaido and Nakae, 1979; Nikaido and Vaara, 1985). With such strict physiological regulation of access by molecules to the periplasm it may appear, at first glance, inconceivable that ALD should work unless the ligands employed are at or below the 650 Da exclusion limit or are analogues of normally permeant compounds. However, the inventors have shown that ligands at least 2000 Da in size can diffuse into the periplasm. Such diffusion can be aided by one or more treatments of a bacterial cell, thereby rendering the outer membrane more permeable, as is described herein below.

## II. Ligand Access to the Bacterial Periplasm

Certain classes of hydrophobic antibiotics, larger than the 650 Da exclusion limit, can diffuse through the bacterial outer membrane itself, independent of membrane porins (Farmer *et al.*, 1999). The process may actually permeabilize the membrane on so doing (Jouenne and Junter, 1990). Such a mechanism has been adopted to selectively label the periplasmic loops of a cytoplasmic membrane protein *in vivo* with a polymyxin B nonapeptide (Wada *et al.*, 1999). Also, certain long chain phosphate polymers (100 Pi) appear to bypass the normal molecular sieving activity of the outer membrane altogether (Rao and Torriani, 1988). However, such conditions generally lead to a decrease in cell viability. Maintaining the cells in a viable state is essential for library screening applications since non-viable cells cannot be propagated.

The inventors have defined conditions that lead to the permeation of ligands into the periplasm without loss of viability or release of the expressed proteins from the cells. As a result, cells expressing binding protein can be fluorescently labeled simply by incubating with a solution of fluorescently labeled ligand. The inventors have observed marked differences in labeling efficiencies of different strains of bacterial host cells. It has been shown previously that increased permeability due to OmpF overexpression was caused by the absence of a histone like protein resulting in a decrease in the amount of a negative regulatory mRNA for OmpF translation (Painbeni *et al.*, 1997). Also, DNA replication and chromosomal segregation is known to rely on intimate contact of the replisome with the inner membrane, which itself contacts the outer membrane at numerous points. That the FACS optimal ABLEC strain has mutations altering plasmid copy number is thus, noteworthy.

The inventors also noticed that treatments such as hyperosmotic shock can improve labeling significantly. It is known that many agents including, calcium ions (Bukau *et al.*, 1985) and even Tris buffer (Irvin *et al.*, 1981) alter the permeability of the outer-membrane. Further, the inventors found that phage infection stimulates the labeling process. Both the filamentous phage inner membrane protein pIII and the large multimeric outer membrane protein pIV can alter membrane permeability (Boeke *et al.*, 1982) with mutants in pIV known to improve access to maltodextrins normally excluded (Marciano *et al.*, 1999). Using

the techniques of the invention comprising a judicious combination of strain, salt and phage, the inventors surpassed the highest fluorescent signal reported even for cell surface display of the antidigoxin antibody scFv (Daugherty *et al.*, 1999). This result demonstrated that the anchor-less display methodology allows for excellent ligand-dependent labeling of cells. Cells labeled with a fluorescent ligand can then be easily isolated from cells that express non-binding protein mutants using flow cytometry or other related techniques.

### **III. Periplasmic Peptide Expression**

In one embodiment of the invention, bacterial cells are provided expressing candidate molecules in the periplasm of the bacteria. An advantage of the instant invention is that, unlike prior art phage display techniques, it is not necessary that the candidate molecule be surface-bound, thereby limiting the potential for effects due to surface interactions with the candidate molecule, or limitations in the expression thereof. Thus, the invention employs "anchor-less display." The general scheme behind the technique of the invention is the advantageous expression of a heterogeneous collection of peptides in soluble form in the periplasm.

Methods that may be employed with the current invention for the expression of heterologous proteins in the periplasm of Gram negative bacteria are well known in the art (see, for example, U.S. Patents 5,646,015 and 5,759,810, the disclosures of which are incorporated herein by reference in their entirety). In such techniques, bacterial-encoded heterologous proteins can be directed across the inner membrane of the bacterial cell envelope, into the space between the inner and the outer membrane known as the periplasm. For example, when a protein is expressed as a fusion protein having an *E. coli*-recognized peptide or "signal peptide" attached to its N-terminus, the desired protein is secreted into the periplasm. Signal peptides that could potentially be employed with the invention are well known in the art and include, for example, those described by Watson (1984), Oka *et al.*, (1985), Hsiung *et al.*, (1986) and EP 177,343, each of the disclosures of which are specifically incorporated herein by reference in their entirety.

In phage display, a gene encoding a protein of interest is commonly linked to the amino-terminal domain of the gene III coat protein of the filamentous phage M13, or another surface-associated molecule. The fusion is mutated to form a library of structurally related fusion proteins that are expressed in low quantity on the surface of phagemid candidates. For example, U.S. Patent 5,571,698 describes directed evolution using an M13 phagemid system. However, in the instant invention, fusion to the gene III coat protein is not necessary, as the protein is expressed in soluble form in the periplasm. Instead, it may be desirable to create a fusion protein of the candidate periplasmic-expressed binding protein or antibody with a signal sequence directing expression of that protein in the periplasm. As such, techniques for the creation of heterogeneous collections of candidate molecules, well known to those of skill in the art in conjunction with phage display, could be adapted for use with the invention. Those of skill in the art will recognize that such adaptations will include the use of bacterial elements for expression and secretion of candidate molecules into the periplasm, including, promoter, enhancers or leader sequences. The current invention provides the advantage relative to phage display of not requiring the creation of fusions with surface-associated molecules, as required in standard display protocols, which may be poorly expressed or may be deleterious to the host cell.

Examples of techniques that could be employed in conjunction with the invention for expression of candidate binding proteins and/or antibodies in the periplasm include the techniques for expression of immunoglobulin heavy chain libraries described in U.S. Patent 5,824,520. In this technique, a single chain antibody library is generated by creating highly divergent, synthetic hypervariable regions. Similar techniques for antibody display are given by U.S. Patent 5,922,545.

and subjected to potential inhibition or modification by target ligands. Those bacteria in which modifications have taken place can then be separated from those that have not. By employing FACS screening technology, the general progress of a reaction could similarly be efficiently monitored.

#### **IV. Screening Candidate Molecules**

The present invention further comprises methods for identifying molecules capable of binding a target ligand. The molecules screened may comprise large libraries of diverse candidate substances, or, alternatively, may comprise particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to bind the target ligand. In a preferred embodiment of the invention, the candidate molecule is an antibody, or a fragment or portion thereof. In other embodiments of the invention, the candidate molecule may be another binding protein or an enzyme.

To identify a candidate molecule capable of binding a target ligand in accordance with the invention, one may carry out the steps of: providing a population of Gram negative bacterial cells comprising candidate molecules expressed in the periplasm of the bacteria; admixing the bacteria and at least a first labeled target ligand capable of diffusing into the periplasm of the bacteria; and identifying at least a first bacterium expressing a molecule capable of binding the target ligand.

In the aforementioned method, the binding between the candidate molecule and the ligand will prevent the diffusing out of the cell. In this way, multiple molecules of the labeled ligand will be retained in the periplasm of the bacterium. The labeling may then be used to isolate the cell expressing the molecule capable of binding the target ligand, and in this way, the gene encoding the molecule isolated. The molecule capable of binding the target ligand may then be produced in large quantities using *in vivo* or *ex vivo* expression methods, and then used for any desired application, for example, for diagnostic or therapeutic applications, as described below.

As used herein the term "candidate molecule" or "candidate polypeptide" refers to any molecule or polypeptide that may potentially have affinity for a target ligand. The candidate substance may be a protein or fragment thereof, including a small molecule. The candidate molecule may in one embodiment of the invention, comprise an antibody sequence or fragment thereof. Such sequences may be particularly designed for the likelihood that they will bind a target ligand.

Binding proteins or antibodies isolated in accordance with the invention also may help ascertain the structure of a target ligand. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for binding the target ligand. Such libraries could be provided by way of nucleic acids encoding the small molecules or bacteria expressing the molecules.

## **V. Screening of Enzyme Libraries**

Yet another aspect of the present invention relates to the isolation of enzyme catalysts capable of catalyzing the conversion of a reaction substrate to a product. The inventors have discovered that a number of fluorescent substrates for enzymatic reactions can permeate into the periplasmic space of *E. coli*. Cells that express an enzyme capable of reacting with such a substrates produce fluorescent products in direct proportion to the catalytic activity of the protein. The inventors have further found that the fluorescent product of the enzymatic reaction is retained within the cell. As a result, cells become fluorescently stained in

proportion to the catalytic activity of the expressed enzyme and can be sorted from a population of mutant proteins by FACS.

Flow cytometry is well suited for the analysis of enzyme activity and kinetics at the single cell level. The inventors have discovered that many fluorescent substrates that normally cannot permeate into the cytoplasm can nonetheless freely diffuse into the periplasm of bacterial cells. Cells expressing an enzyme in the bacterial periplasm are thus capable of converting the substrate into the respective fluorescent product. The inventors further discovered, unexpectedly, that following the enzymatic reaction the fluorescent product is selectively retained within the periplasm and thus the cell becomes fluorescent and can be isolated by FACS.

In the case of the LysoSensor Green DND-189 in the presence of 1mM 4-nitrophenyl butyrate reaction, it is not a fluorescent product that is being detected. Rather, the LysoSensor Green DND-189 is a pH-sensitive dye, and as the enzyme catalyzes the conversion of 4-nitrophenyl butyrate to 4-nitrophenol and butyric acid in the periplasm, the pH drops in unbuffered solution. This drop in pH is being detected as increased fluorescence by the LysoSensor Green DND-189, which remains associated with the cell via electrostatic attraction. Here, the LysoSensor Green DND-189 becomes protonated at lower pH and thus positively-charged, so it associates with the negatively-charged bacterial outer membrane long enough to allow FACS isolation.

### **1. Cloning of Binding Protein Coding Sequences**

The binding affinity of an antibody or another binding protein can, for example, be determined by the Scatchard analysis of Munson & Pollard (1980). After a bacterial cell is identified that produces molecules of the desired specificity, affinity, and/or activity, the corresponding coding sequence may be cloned. In this manner, DNA encoding the molecule can be isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the antibody or binding protein).

Once isolated, the antibody or binding protein DNA may be placed into expression vectors, which can then be transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of binding protein in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (Morrison, *et al.*, 1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" binding proteins are prepared that have the desired binding specificity.

Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for the target ligand and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

## **2. Maximization of Protein Affinity for Ligands**

In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks *et al.*, 1992). In this method, the affinity of "primary" human antibodies obtained in accordance with the invention could be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody

fragments with affinities in the nM range. A strategy for making very large antibody repertoires was described by Waterhouse *et al.*, (1993), and the isolation of a high affinity human antibody directly from such large phage library was reported by Griffith *et al.*, (1994). Gene shuffling also can be used to derive human antibodies from rodent antibodies, where the human antibody has similar affinities and specificities to the starting rodent antibody. According to this method, which is also referred to as "epitope imprinting", the heavy or light chain V domain gene of rodent antibodies obtained by the phage display technique is replaced with a repertoire of human V domain genes, creating rodent-human chimeras. Selection on the antigen results in isolation of human variable regions capable of restoring a functional antigen-binding site, *i.e.* the epitope governs (imprints) the choice of partner. When the process is repeated in order to replace the remaining rodent V domain, a human antibody is obtained (see PCT patent application WO 93/06213, published Apr. 1, 1993). Unlike traditional humanization of rodent antibodies by CDR grafting, this technique provides completely human antibodies, which have no framework or CDR residues of rodent origin.

### **3. Labeled Ligands**

In one embodiment of the invention, an antibody or binding protein is isolated which has affinity for a labeled ligand. Such a labeled ligand is, in one embodiment of the invention, preferably less than 50,000 Da in size in order to allow efficient diffusion of the ligand into the bacterial periplasm. As indicated above, it will typically be desired in accordance with the invention to provide a ligand which has been labeled with one or more detectable agent(s). This can be carried out, for example, by linking the ligand to at least one detectable agent to form a conjugate. For example, it is conventional to link or covalently bind or complex at least one detectable molecule or moiety. A "label" or "detectable label" is a compound and/or element that can be detected due to specific functional properties, and/or chemical characteristics, the use of which allows the ligand to which it is attached to be detected, and/or further quantified if desired. Examples of labels which could be used with the invention include, but are not limited to, enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, luminescent molecules, photoaffinity molecules, colored particles or ligands, such as biotin.

In a preferred embodiment of the invention, a visually-detectable marker is used such that automated screening of cells for the label can be carried out. In particular, fluorescent labels are preferred in that they allow use of FACS for isolation of cells expressing a desired binding protein or antibody. Examples of agents that may be detected by visualization with an appropriate instrument are known in the art, as are methods for their attachment to a desired ligand (see, *e.g.*, U.S. Patents 5,021,236; 4,938,948; and 4,472,509, each incorporated herein by reference). Such agents can include paramagnetic ions; radioactive isotopes; fluorochromes; NMR-detectable substances and substances for X-ray imaging. Types of fluorescent labels that may be used with the invention will be well known to those of skill in the art and include, for example, Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red.

Examples of paramagnetic ions that could be used as labels include ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and/or erbium (III). Ions useful in other contexts include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Another type of ligand conjugates contemplated in the present invention are those where the ligand is linked to a secondary binding molecule and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of such enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. In such instances, it will be desired that cells selected remain viable. Preferred secondary binding ligands are biotin and/or avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and are

described, for example, in U.S. Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241; each incorporated herein by reference.

Molecules containing azido groups also may be used to form covalent bonds to proteins through reactive nitrene intermediates that are generated by low intensity ultraviolet light (Potter & Haley, 1983). In particular, 2- and 8-azido analogues of purine nucleotides have been used as site-directed photoprobes to identify nucleotide-binding proteins in crude cell extracts (Owens & Haley, 1987; Atherton *et al.*, 1985). The 2- and 8-azido nucleotides have also been used to map nucleotide-binding domains of purified proteins (Khatoon *et al.*, 1989; King *et al.*, 1989; and Dholakia *et al.*, 1989) and may be used as ligand binding agents.

Labeling can be carried out by any of the techniques well known to those of skill in the art. For instance, ligands can be labeled by contacting the ligand with the desired label and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Similarly, a ligand exchange process could be used. Alternatively, direct labeling techniques may be used, *e.g.*, by incubating the label, a reducing agent such as  $\text{SnCl}_2$ , a buffer solution such as sodium-potassium phthalate solution, and the ligand. Intermediary functional groups on the ligand could also be used, for example, to bind labels to a ligand in the presence of diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

Other methods are also known in the art for the attachment or conjugation of a ligand to its conjugate moiety. Some attachment methods involve the use of an organic chelating agent such as a diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3 $\alpha$ -6 $\alpha$ -diphenylglycouril-3 attached to the ligand (U.S. Patents 4,472,509 and 4,938,948, each incorporated herein by reference). Ligands also may be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers can be prepared in the presence of these coupling agents or by reaction with an isothiocyanate. In U.S. Patent 4,938,948, imaging of breast tumors is achieved using monoclonal antibodies and the detectable imaging moieties are bound to the antibody using

linkers such as methyl-p-hydroxybenzimidate or N-succinimidyl-3-(4-hydroxyphenyl)propionate.

## V. Illustrative Use of the Invention

The examples described herein demonstrate the use of the invention for the affinity maturation of the anti-cardiac glycoside scFv 26.10, and the de novo isolation of antibodies from large repertoire libraries. Importantly the current invention results in the isolation of protein variants that are missed by other protein library screening technologies such as phage display. As discussed above, most scFv are toxic to the host cell when targeted to the periplasm. Although reasonable expression levels of such molecules may be obtained by altering culture conditions, provision of chaperones and foldases *etc.*, the host cells are often not recoverable after expression. FACS selection with the anchor-less-display (ALD) techniques of the invention requires a high enough expression level for a strong FACS signal but not so high as to irreversibly damage the cell and prevent its isolation. In contrast, phage display imposes little selection for expression. As a result, proteins isolated from a phage display library screening program are often very difficult to produce in sufficient quantities for further characterization. The advent of highly expressible framework-based libraries (Knappick *et al.*, 2000) should help circumvent the expression versus viability problem. The high sort-rates of current FACS machines generally enable the screening of very large libraries ( $10^9$ - $10^{10}$ ) and can completely circumvent phage or cell surface display.

The ability to specifically label periplasmic expressed proteins with appropriate fluorescent ligands also has applications other than library screening. Specifically labeling with fluorescent ligands and flow cytometry can be used for monitoring production during protein manufacturing. While flow cytometry has been used previously for the analysis of bacterial cells, it has not been used for the specific labeling and quantitation of periplasmic proteins. However, a large number of commercially important proteins including IGF-1 several interleukins, enzymes such as urokinase-type plasminogen activator, antibody fragments, inhibitors (*e.g.*, Bovine pancreatic trypsin inhibitor) are expressed in recombinant bacteria in a form secreted into the periplasmic space. The level of production of such proteins within each cell in a culture can be monitored by utilizing an appropriate fluorescent

ligand and flow cytometric analysis, according to the techniques taught by the present invention.

Generally, monitoring protein expression requires cell lysis and detection of the protein by immunological techniques or following chromatographic separation. However, ELISA or western blot analysis is time-consuming and does not provide information on the distribution of expression among a cell population and cannot be used for on-line monitoring (Thorstenson *et al.*, 1997; Berrier *et al.*, 2000). In contrast, FACS labeling is rapid and simple and can well be applied to online monitoring of industrial size fermentations of recombinant proteins expressed in gram-negative bacteria. Similarly, the invention could be used to monitor the production of a particular byproduct of a biological reaction. This also could be used to measure the relative concentration or specific activity of an enzyme expressed *in vivo* in a bacterium or provided *ex vivo*. The passive and instant nature of ALD provides the advantage of allowing instant analysis of a population of cells with direct observations rather than relying on extensive indirect protocols.

Once a ligand-binding protein, such as an antibody, has been isolated in accordance with the invention, it may be desired to link the molecule to at least one agent to form a conjugate to enhance the utility of that molecule. For example, in order to increase the efficacy of antibody molecules as diagnostic or therapeutic agents, it is conventional to link or covalently bind or complex at least one desired molecule or moiety. Such a molecule or moiety may be, but is not limited to, at least one effector or reporter molecule. Effector molecules comprise molecules having a desired activity, *e.g.*, cytotoxic activity. Non-limiting examples of effector molecules which have been attached to antibodies include toxins, anti-tumor agents, therapeutic enzymes, radio-labeled nucleotides, antiviral agents, chelating agents, cytokines, growth factors, and oligo- or poly-nucleotides. By contrast, a reporter molecule is defined as any moiety which may be detected using an assay. Techniques for labeling such a molecule are known to those of skill in the art and have been described herein above.

Labeled binding proteins such as antibodies which have been prepared in accordance with the invention may also then be employed, for example, in immunodetection methods for binding, purifying, removing, quantifying and/or otherwise generally detecting biological components such as protein(s), polypeptide(s) or peptide(s). Some immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle MH and Ben-Zeev O, 1999; Gulbis B and Galand P, 1993; and De Jager R *et al.*, 1993, each incorporated herein by reference. Such techniques include binding assays such as the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art.

The ligand-binding molecules, including antibodies, prepared in accordance with the present invention may also, for example, in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Abbondanzo *et al.*, 1990).

## **VI. Automated Screening with FACS**

In one embodiment of the invention, fluorescence activated cell sorting (FACS) screening or other automated flow cytometric techniques may be used for the efficient isolation of a bacterial cell comprising a labeled ligand bound to a candidate molecule in the periplasm of the bacteria. Instruments for carrying out FACS are known to those of skill in the art and are commercially available to the public. Examples of such instruments include FACS Star Plus, FACScan and FACSsort instruments from Becton Dickinson (Foster City, Calif.) Epics C from Coulter Epics Division (Hialeah, Fla.) and MoFlo from Cytomation (Colorado Springs, Co).

Flow cytometric techniques in general involve the separation of cells or other particles in a liquid sample. Typically, the purpose of flow cytometry is to analyze the separated particles for one or more characteristics thereof, for example, presence of a labeled ligand or other molecule. The basis steps of flow cytometry involve the direction of a fluid sample through an apparatus such that a liquid stream passes through a sensing region. The particles should pass one at a time by the sensor and are categorized base on size, refraction, light scattering, opacity, roughness, shape, fluorescence, *etc.*

Rapid quantitative analysis of cells proves useful in biomedical research and medicine. Apparati permit quantitative multiparameter analysis of cellular properties at rates of several thousand cells per second. These instruments provide the ability to differentiate among cell types. Data are often displayed in one-dimensional (histogram) or two-dimensional (contour plot, scatter plot) frequency distributions of measured variables. The partitioning of multiparameter data files involves consecutive use of the interactive one- or two-dimensional graphics programs.

Quantitative analysis of multiparameter flow cytometric data for rapid cell detection consists of two stages: cell class characterization and sample processing. In general, the process of cell class characterization partitions the cell feature into cells of interest and not of interest. Then, in sample processing, each cell is classified in one of the two categories according to the region in which it falls. Analysis of the class of cells is very important, as high detection performance may be expected only if an appropriate characteristic of the cells is obtained.

Not only is cell analysis performed by flow cytometry, but so too is sorting of cells. In U.S. Patent 3,826,364, an apparatus is disclosed which physically separates particles, such as functionally different cell types. In this machine, a laser provides illumination which is focused on the stream of particles by a suitable lens or lens system so that there is highly localized scatter from the particles therein. In addition, high intensity source illumination is directed onto the stream of particles for the excitation of fluorescent particles in the stream. Certain particles in the stream may be selectively charged and then separated by deflecting

them into designated receptacles. A classic form of this separation is *via* fluorescent-tagged antibodies, which are used to mark one or more cell types for separation.

Other methods for flow cytometry can be found in U.S. Patents 4,284,412; 4,989,977; 4,498,766; 5,478,722; 4,857,451; 4,774,189; 4,767,206; 4,714,682; 5,160,974; and 4,661,913, each of the disclosures of which are specifically incorporated herein by reference.

## **VII. Nucleic Acid-Based Expression Systems**

Nucleic acid-based expression systems may find use, in certain embodiments of the invention, for the expression of recombinant proteins. For example, one embodiment of the invention involves transformation of Gram negative bacteria with the coding sequences of candidate antibody or other binding proteins having affinity for a selected ligand and the expression of such candidate molecules in the periplasm of the Gram negative bacteria. In other embodiments of the invention, expression of such coding sequences may be carried, for example, in eukaryotic host cells for the preparation of isolated binding proteins having specificity for the target ligand. The isolated protein could then be used in one or more therapeutic or diagnostic applications.

### **1. Methods of Nucleic Acid Delivery**

Certain aspects of the invention may comprise delivery of nucleic acids to target cells. For example, bacterial host cells may be transformed with nucleic acids encoding candidate molecules potentially capable binding a target ligand. In particular embodiments of the invention, it may be desired to target the expression to the periplasm of the bacteria. Transformation of eukaryotic host cells may similarly find use in the expression of various candidate molecules identified as capable of binding a target ligand.

Suitable methods for nucleic acid delivery for transformation of a cell are believed to include virtually any method by which a nucleic acid (*e.g.*, DNA) can be introduced into such a cell, or even an organelle thereof. Such methods include, but are not limited to, direct delivery of DNA such as by injection (U.S. Patents 5,994,624, 5,981,274, 5,945,100, 5,780,448, 5,736,524, 5,702,932, 5,656,610, 5,589,466 and 5,580,859, each incorporated

herein by reference), including microinjection (Harlan and Weintraub, 1985; U.S. Patent 5,789,215, incorporated herein by reference); by electroporation (U.S. Patent 5,384,253, incorporated herein by reference); by calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990); by using DEAE-dextran followed by polyethylene glycol (Gopal, 1985); by direct sonic loading (Fechheimer *et al.*, 1987); by liposome mediated transfection (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987; Wong *et al.*, 1980; Kaneda *et al.*, 1989; Kato *et al.*, 1991); by microprojectile bombardment (PCT Application Nos. WO 94/09699 and 95/06128; U.S. Patents 5,610,042; 5,322,783 5,563,055, 5,550,318, 5,538,877 and 5,538,880, and each incorporated herein by reference); by agitation with silicon carbide fibers (Kaeppeler *et al.*, 1990; U.S. Patents 5,302,523 and 5,464,765, each incorporated herein by reference); by *Agrobacterium*-mediated transformation (U.S. Patents 5,591,616 and 5,563,055, each incorporated herein by reference); or by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993; U.S. Patents 4,684,611 and 4,952,500, each incorporated herein by reference); by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985). Through the application of techniques such as these, organelle(s), cell(s), tissue(s) or organism(s) may be stably or transiently transformed.

**a. Electroporation**

In certain embodiments of the present invention, a nucleic acid is introduced into a cell *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent 5,384,253, incorporated herein by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding.

**b. Calcium Phosphate**

In other embodiments of the present invention, a nucleic acid is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also

manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

## 2. Vectors

Vectors may find use with the current invention, for example, in the transformation of a gram negative bacterium with a nucleic acid sequence encoding a candidate polypeptide which one wishes to screen for ability to bind a target ligand. In one embodiment of the invention, an entire heterogeneous "library" of nucleic acid sequences encoding target polypeptides may be introduced into a population of bacteria, thereby allowing screening of the entire library. The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art may construct a vector through standard recombinant techniques, which are described in Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both of which references are incorporated herein by reference.

The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra*.

**a. Promoters and Enhancers**

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated that the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. One example of such promoter that may be used with the invention is the *E. coli* arabinose promoter. Those of skill in the art of molecular biology generally are familiar with the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

**b. Initiation Signals and Internal Ribosome Binding Sites**

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

**c. Multiple Cloning Sites**

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) “Restriction enzyme digestion” refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is understood by those of skill in

the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

#### **d. Termination Signals**

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments, a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, rhp dependent or rho independent terminators. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

#### **e. Origins of Replication**

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

#### **f. Selectable and Screenable Markers**

In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker

is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

### **3. Host Cells**

As used herein, the terms “cell,” “cell line,” and “cell culture” may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a prokaryotic or eukaryotic cell, and it includes any transformable organism that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be “transfected” or “transformed,” which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

In particular embodiments of the invention, a host cell is a gram negative bacterial cell. These bacteria are suited for use with the invention in that they possess a periplasmic space between the inner and outer membrane. As such, any other cell with such a periplasmic space could be used in accordance with the invention. Examples of Gram negative bacteria that may find use with the invention may include, but are not limited to, *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Salmonella typhimurium*, *Shigella flexneri*, *Haemophilus influenza*, *Bordetella pertussi*, *Erwinia amylovora*, *Rhizobium sp.* The Gram negative bacterial cell may be still further defined as bacterial cell which has been transformed with the coding sequence of a candidate polypeptide capable of binding a selected ligand. The polypeptide will be expressed in the periplasmic space, and may comprise an antibody coding sequence or another sequence. One means for expression of the polypeptide in the periplasm is by attaching a leader sequence to the polypeptide capable of causing such directing.

Numerous prokaryotic cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials ([www.atcc.org](http://www.atcc.org)). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5 $\alpha$ , JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE<sup>®</sup> Competent Cells and SOLOPACK<sup>™</sup> Gold Cells (STRATAGENE<sup>®</sup>, La Jolla). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells for bacteriophage.

Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either a eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector. Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described

host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

#### **4. Expression Systems**

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Such systems could be used, for example, for the production of a polypeptide product identified in accordance with the invention as capable of binding a particular ligand. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

Other examples of expression systems include STRATAGENE<sup>®</sup>'s COMPLETE CONTROL<sup>™</sup> Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from INVITROGEN<sup>®</sup>, which carries the T-REX<sup>™</sup> (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. INVITROGEN<sup>®</sup> also provides a yeast expression system called the *Pichia methanolica* Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

#### **5. Candidate Binding Proteins and Antibodies**

In certain aspects of the invention, candidate antibodies or other recombinant proteins potentially capable of binding a target ligand are expressed in the periplasm of a host bacterial cell. By expression of a heterogeneous population of such antibodies, those antibodies having a high affinity for a target ligand may be identified. The identified

antibodies may then be used in various diagnostic or therapeutic applications, as described herein.

As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. The term "antibody" is also used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, F(ab')<sub>2</sub>, single domain antibodies (DABs), Fv, scFv (single chain Fv), and engineering multivalent antibody fragments such as dibodies, tribodies and multibodies. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Once an antibody having affinity for a target ligand is identified, the antibody may be purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Fragments of such antibodies can be obtained from the antibodies so produced by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer.

A molecular cloning approach comprises one suitable method for the generation of a heterogeneous population of candidate antibodies that may then be screened in accordance with the invention for affinity to target ligands. In one embodiment of the invention, combinatorial immunoglobulin phagemid can be prepared from RNA isolated from the spleen of an animal. By immunizing an animal with the ligand to be screened, the assay may be targeted to the particular antigen. The advantages of this approach over conventional techniques are that approximately 10<sup>4</sup> times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination which further increases the chance of finding appropriate antibodies.

## VIII. Manipulation and Detection of Nucleic Acids

In certain embodiments of the invention it may be desired to employ one or more techniques for the manipulation and/or detection of nucleic acids. Such techniques may include, for example, the preparation of vectors for transformation of host cells as well as methods for cloning selected nucleic acid segments from a transgenic cells. Methodology for carrying out such manipulations will be well known to those of skill in the art in light of the instant disclosure.

### 1. Amplification of Nucleic Acids

Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis may be performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

Pairs of primers designed to selectively hybridize to nucleic acids corresponding to a selected nucleic acid sequence are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid

synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even via a system using electrical and/or thermal impulse signals (Affymax technology; Bellus, 1994).

A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1988, each of which is incorporated herein by reference in their entirety.

A reverse transcriptase PCR<sup>TM</sup> amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known (see Sambrook *et al.*, 1989). Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent 5,882,864.

Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCR<sup>TM</sup> and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patents 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574,

5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

PCT Application WO 89/06700 (incorporated herein by reference in its entirety) discloses a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

## 2. Other Assays

Other methods for genetic screening may be used within the scope of the present invention, for example, to detect mutations in genomic DNA, cDNA and/or RNA samples. Methods used to detect point mutations include denaturing gradient gel electrophoresis ("DGGE"), restriction fragment length polymorphism analysis ("RFLP"), chemical or enzymatic cleavage methods, direct sequencing of target regions amplified by PCR<sup>TM</sup> (see above), single-strand conformation polymorphism analysis ("SSCP") and other methods well known in the art.

One method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA or RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single or multiple base point mutations.

U.S. Patent 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

Other investigators have described the use of RNase I in mismatch assays. The use of RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is reported to cleave three out of four known mismatches. Others have described using the MutS protein or other DNA-repair enzymes for detection of single-base mismatches.

Alternative methods for detection of deletion, insertion or substitution mutations that may be used in the practice of the present invention are disclosed in U.S. Patents 5,849,483, 5,851,770, 5,866,337, 5,925,525 and 5,928,870, each of which is incorporated herein by reference in its entirety.

### 3. Kits

All the essential materials and/or reagents required for detecting a target ligand may be provided by the invention and may be assembled together in a kit. This generally will comprise an antibody or binding protein prepared in accordance with the invention and designed to have affinity specifically to a target ligand. Also included may be buffers to provide the necessary mixture for binding to the ligand, as well as labeling means for detecting the binding. Such kits may also include enzymes and other reagents suitable for detection of specific ligands. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or enzyme as well as for antibody or binding protein.

## IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### EXAMPLE 1

#### Fluorescence Detection and Enrichment of Cells Expressing

#### scFv Antibodies in the Periplasm

The 26-10 scFv antibody binds with high affinity to cardiac glycosides such as digoxin and digoxigenin ( $K_D$  of the purified antibodies for digoxin and digoxigenin are  $0.9 \pm$

0.2 x 10<sup>-9</sup> M<sup>-1</sup> and 2.4±0.4 x10<sup>-9</sup> M<sup>-1</sup>, respectively, Chen *et al.*, 1999). The 26-10 scFv and its variants have been used extensively as a model system to understand the effect of mutations in the CDRs and in the framework regions on hapten binding (Schilbach *et al.*, 1992; Short *et al.*, 1995; Daugherty *et al.*, 1998, 2000; Chen *et al.*, 1999). A derivative of the 26-10 scFv was expressed under the *E. coli* arabinose promoter and with the *pelB* leader peptide that allows secretion in the *E. coli* periplasm. The resulting plasmid vector (pBAD30pelB-Dig) was transformed in the *ara*<sup>-</sup> *E. coli* strain LMG194 and protein synthesis was induced with 0.2% w/v arabinose. It was observed that upon incubation with 200 nM of digoxigenin-BODIPY™, cells that had been grown at 25°C became strongly fluorescent and the fluorescence signal was retained even after extensive washing to remove non-specifically bound ligand. The labeling of the cells with a probe having a M.W. which is significantly higher than the generally accepted size limit of about 600 Da for the permeation of hydrophilic solutes in the periplasm (Decad and Nikaido, 1976) raised the possibility that the fluorescence signal was mainly due to non-viable, permeabilized cells. However, staining with the viability stain propidium iodide, which binds specifically to membrane damaged cells by virtue of intercalating with the normally inaccessible nucleic acids, revealed that >90% of the cells were not permeable to the dye. This is similar to the proportion of intact cells in control *E. coli* cultures harvested in late exponential phase.

Cells expressing the 26-10 antibody in the periplasm could be enriched from a large excess of *E. coli* transformed with vector alone in a single round of sorting. Specifically, LMG194 (pBAD30pelB-Dig) were mixed with a 10,000 fold excess of *E. coli* containing empty vector (pBAD30). The former cells are resistant to both ampicillin and chloramphenicol (amp<sup>r</sup>, Cm<sup>r</sup>) whereas the latter are resistant to ampicillin only (amp<sup>r</sup>). 4 hours after induction with 0.2% w/v arabinose, the cells were then labeled with 100nM digoxigenin-BODIPY™ for 1 hour and fluorescent cells were isolated by FACS. Following re-growth of the sorted cells and re-labeling as above, the population exhibited a five to eight-fold increase in the mean fluorescence intensity (FL1=20 vs FL1=4 for the pre-sort cell mixture). The fraction of scFv-expressing clones in the enriched population was estimated from the number of amp<sup>r</sup> clones that were also Cm<sup>r</sup>. 80% of the amp<sup>r</sup> colonies were also Cm<sup>r</sup>



indicating that fluorescence labeling and cell sorting gave an enrichment of well over 1,000-fold in a single round

## EXAMPLE 2

### Antibody Affinity Maturation

Short *et al.*, (1995) isolated a 26-10 mutant, designated A4-19, having an equilibrium dissociation constant ( $K_D$ ) for digoxin of 300 pM as measured by surface plasmon resonance. A4-19 contains 3 amino acid substitutions in heavy chain CDR1 ( $V_H$ :T30->P,  $V_H$ :D31->S and  $V_H$ :M34->Y). It was examined whether mutants with increased binding affinity can be obtained by periplasmic expression/FACS screening even when starting with an antibody that already exhibits very tight binding. Three light chain CDR3 residues that make contact ( $V_L$ :T91,  $V_L$ :P96) or are in close proximity to ( $V_L$ :V94) the digoxin hapten (Jeffrey *et al.*, 1993) were randomized using an NNS (S=G or C) strategy (Daugherty *et al.* 1998). A library of  $2.5 \times 10^6$  transformants expressed in the periplasm *via* the *pelB* leader was generated and screened using two rounds of FACS (FIG. 2). In the first round of screening, cells labeled with 100 nM of the fluorescent probe were washed once with PBS and sorted using recovery mode in which the instrument collects all fluorescent events even if a non-fluorescent particle is detected in the same element of fluid as a fluorescent particle. Operation in recovery mode provided a better assurance that very rare cells would be collected but at the expense of purity.

Collected cells were re-grown, labeled, washed and then incubated with a 50-fold excess (50  $\mu$ M) of free digoxin for various times (15 min to 90 min). Cells that retained the desired level of fluorescence were isolated by sorting using exclusion mode, in which, coincident fluorescent and non-fluorescent events were rejected and thus a higher degree of purity was obtained. The rate of fluorescence decay for the pool of cells obtained following incubation with non-fluorescent competitor for various times was measured. A slightly faster rate compared to the starting A4-19 antibody was observed for the earlier time points (<60 minutes incubation with competitor) but the rate was reduced for the 60 min and 90 min populations. 5 random clones from the cell population obtained after 60 min of competition and 13 clones from the 90 min pool were picked at random and sequenced (Table 1). A

strong sequence consensus was clearly evident. The hapten binding kinetics of the purified antibodies were determined by SPR and the results are shown in Table 1. The corresponding amino acid sequences are given by SEQ ID NOs:1-12. It should be noted that upon purification and analysis by gel filtration FPLC none of the mutants was found to dimerize. All of the mutants examined displayed association rate constants ( $k_{on}$ ) indistinguishable from that of the starting A4-19 antibody ( $0.9 \pm 0.2 \times 10^6 \text{ M}^{-1}$ ). The  $k_{diss}$  of the clones isolated after 60 min of competition were the same or faster than that of A4-19. Clones isolated after 90 minutes of competition exhibited slower  $k_{diss}$  in solution. One clone, 90.3, exhibited a 2-fold slower dissociation rate constant resulting in a  $K_D$  of 150 pM. Thus, the library screening methodology of the invention allowed specific labeling to isolate a better mutant, even when starting with an antibody that already exhibited a sub-nanomolar  $K_D$ . Interestingly, but not surprisingly, the effect of the three heavy chain CDR1 mutations present in 4-19 and the two mutations in residues 94 and 96 of the light chain were additive.

**Table 1.** Heavy and light chain CDR3 amino acid sequences (SEQ ID NOs: 1-12) of mutants isolated by 60min (clones 60.1-60.4 and 90 minutes (clones 90.1-90.6) off-rate selection. Number of identical clones shown in parenthesis. ND: Not Determined.

	<b>Light Chain Sequence</b>	<b>Off-rate / s</b>
Wild Type 26-10 scFv	90.....96 Q T T H V P P	8.4 x 10 <sup>-4</sup>
A14-9	Q T T H V P P	2.7 x 10 <sup>-4</sup>
60.1 (1 clone)	Q T T H S P A	5.5 x 10 <sup>-4</sup>
60.2 (2)	Q T T H L P T	2.8 x 10 <sup>-4</sup>
60.3 (1)	Q T T H T P P	ND
60.4 (1)	Q T T H L P A	ND
90.1 (1)	Q T T H I P T	3.2 x 10 <sup>-4</sup>
90.2 (1)	Q T T H V P P	2.7 x 10 <sup>-4</sup>
90.3 (7)	Q T T H V P A	2.2 x 10 <sup>-4</sup>
90.4 (1)	Q T T H I P A	1.4 x 10 <sup>-4</sup>
90.5 (3)	Q T T H L P A	ND
90.6 (1)	Q T T H V P C	ND

### EXAMPLE 3

#### Maximizing the Fluorescence Signal

The fluorescence intensity of cells expressing scFv antibodies in soluble form in the periplasm was strongly dependent on the *E. coli* strain used and on the growth conditions. With the 26-10 antibody, the maximum fluorescence intensity was obtained when the cells were grown at 25°C. Growth at sub-physiological temperature has several beneficial effects. Expression of scFv at low temperature (*i.e.*, 25°C) facilitates the proper folding of the scFv both directly, by slowing the folding pathway and indirectly by decreasing plasmid copy number to reduce expression load. Indeed, direct expression of scFv at 37°C generally yields little or no soluble protein (for example see Gough *et al.*, 1999). Outer membrane composition is also altered at non-physiological temperatures resulting in increased permeability (Martinez *et al.*, 1999). Rather dramatic differences among various *E. coli* strains were noticed. Among several strains tested, the highest fluorescence intensities were obtained in ABLE<sup>TM</sup>C (FIG. 3). A preliminary analysis of protein expression and outer membrane protein profile in this strain indicated that the higher fluorescent signal was not due to the *pcnB* mutation which reduces the copy number of ColE1 origin plasmids but rather, due to differences in cell envelope protein composition. In fact, the stronger staining of ABLE<sup>TM</sup>C was not related to a higher level of protein expression relative to other strains as deduced by ELISA and Western blotting.

Fluorescent labeling under hyperosmotic conditions, resulted in significantly greater fluorescence (FIG. 4). A 5-7 fold increase in fluorescence was obtained when the cells were incubated in 5x PBS during labeling (a mean FL1 > 150 compared to 20-30 for cells incubated in regular PBS). However, the increased signal came at a cost, as cell viability decreased considerably. Such a decrease in viability may be undesirable when screening highly diverse libraries of proteins, whose expression may already have a deleterious effect on the host cell. Similarly, co-infection with filamentous phages such as M13KO7 induces the phage shock response, which among other things, results in an increase in outer membrane permeability. M13 KO7 infection resulted in a 3-fold increase in the mean fluorescence of the population (FIG. 5). However, as with hyperosmotic shock the viability of the culture, as determined by propidium iodide staining was somewhat decreased.

Labeling of the cells with fluorescent ligand followed by incubation with a large excess of free ligand results in a time-dependent decrease in the mean fluorescence intensity. The rate of the fluorescence decay reflects the dissociation rate of the antibody-antigen complex (Daugherty *et al.*, 2000). For digoxin the rate of fluorescence decay was found to be about 3-4 times slower compared to the dissociation rate measured with the purified antibody using BIACORE. The lower rate of fluorescence decay compared to the dissociation rate of the antibody/antigen complex *in vitro* stems from several effects including the collision frequency between ligands and cells, the concentration of antibody in the periplasm and, of course, the rate of diffusion through the outer membrane (see Martinez *et al.*, (1996) for an analysis of kinetics in the periplasmic space). As may be expected, the ratio of the rate of fluorescence decay in the periplasm relative to the *in vitro* determined  $k_{off}$  rate is antigen dependent.

#### EXAMPLE 4

##### **Analysis and Screening of Repertoire Antibody Libraries by FACS**

Antibodies can be isolated *de novo*, *i.e.*, without animal immunization, by screening large, repertoire libraries that contain a wide variety of antibody sequences. The screening of such large libraries is well established (Nissim *et al.* 1994, Winter *et al.* 1994, Griffith *et al.* 1994, Knappik *et al.* 2000). It was important to establish: (a) How anchor-less display (ALD) compares with the phage display technology in terms of allowing the isolation of high affinity clones and (b) whether ALD can be used to screen highly diverse libraries.

So far all the large antibody repertoire libraries available have been constructed for use with phage display. The inventors discovered that they could take advantage of the fact that libraries constructed for phage display also allow the expression of proteins within the bacterial periplasmic space. In particular, for low protein copy number display on filamentous bacteriophage, recombinant polypeptides are expressed as N-terminal fusions to pIII. During the course of phage biogenesis, pIII fusions are first targeted to the periplasm and anchored in the inner membrane by a small C-terminal portion of pIII. As phage are released, the scFv-pIII fusion is incorporated alongside wild-type pIII at the terminus of the

phage, thereby concluding the assembly process (Rakonjac and Model, 1998; Rakonjac *et al.*, 1999). In the most widely used vectors for phage display an amber codon is placed between the N-terminal scFv and the pIII gene. Thus, in a suitable *E. coli* suppressor strain, full-length scFv-pIII fusion protein is produced for displaying the scFv whereas in a non-suppressor strain only soluble scFv is expressed. The degree of suppression varies with vector and strain but tends to allow only 10% read-through. Thus, as a consequence of the biology of phage display, all amber-codon containing libraries result in a degree of periplasmic expression regardless of host. Hence, it was of great interest to explore whether FACS can aid the isolation of ligand binding proteins from pre-existing, highly diverse, naive libraries (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; Sheets *et al.*, 1998; Pini *et al.*, 1998; de Haard *et al.*, 1999; Knappik *et al.*, 2000; Sblattero and Bradbury, 2000).

Conventional screening of the phage library by phage panning enriched phage expressing scFvs specific for the cardiac glycoside digoxin (FIG. 6A, B) from a naïve antibody repertoire library. The panning process was performed on a BSA conjugate and the screening was performed on an ovalbumin conjugate to reduce the incidence of protein and hapten-protein interface binders. 24 positive isolates from pan 4 shared the same fingerprint and DNA sequencing of 6 clones confirmed the same heavy and light chain sequence ("dig1") with one of six ("dig2") having a unique HCDR3 and LCDR3 combination (FIG. 7). Repeated screening of the phage library both under identical and under different conditions resulted only in the isolation of clones with the same DNA fingerprint.

FACS analysis of the phage rescued in *E. coli* ABLE<sup>TM</sup>C after each round of panning reveals an increase in mean fluorescence at round 3 which mirrors the phage ELISA signals (FIG. 6C). Significant enrichment of binding clones using a single round of FACS was obtained starting with the population obtained from the 3<sup>rd</sup> round of phage panning. This result is consistent with the enrichment profiles obtained during the course of the panning experiment. FACS screening and sorting 10<sup>6</sup> cells from rounds 3, 4 and 5 resulted in the isolation of positive clones at a frequency of 30, 80 and 100% respectively.

Out of 14 clones isolated by FACS from the round 3 population 5 were found to be positive for binding to digoxin. Importantly three of the clones corresponded to a different antibody that was missed by phage panning (herein known as "dig3"). The remaining 2 were the dig1 clone. This result demonstrates that FACS screening of libraries expressed in the periplasmic space and labeled with fluorescent ligands results in the isolation of clones that cannot be isolated by other library screening methodologies.

## EXAMPLE 5

### Materials And Methods

#### A. Strains and plasmids

*E. coli* strains TG1 and HB2151 were provided with the Griffin library. ABLE<sup>TM</sup>C and ABLE<sup>TM</sup>K were purchased from Stratagene and helper phage M13K07 from Pharmacia. A positive control for FACS analysis of a phage display vehicle was constructed by replacing a pre-existing scFv in pHEN2 with the 26.10 scFv to create pHEN2.dig. The negative control was pHEN2.thy bearing the anti-thyroglobulin scFv provided with the Griffin.1 library. The P<sub>tac</sub> vector was a derivative of pIMS120 (Hayhurst, 2000).

#### B. Phage panning

The Griffin.1 library is a semi-synthetic scFv library derived from a large repertoire of human heavy and light chains with part or all of the CDR3 loops randomly mutated and recombined *in vivo* (Griffiths *et al.*, 1994). The library was rescued and subjected to five rounds of panning according to the web-site instruction manual ([www.mrc-cpe.cam.ac.uk/~phage/g1p.html](http://www.mrc-cpe.cam.ac.uk/~phage/g1p.html)), summarized in Example 6, below. Immunotubes were coated with 10µgml<sup>-1</sup> digoxin-BSA conjugate and the neutralized eluates were halved and used to infect either TG-1 for the next round of phage panning, or ABLE<sup>TM</sup> C for FACS analysis.

Eluate titers were monitored to indicate enrichment of antigen binding phage. To confirm reactivity, a polyclonal phage ELISA of purified, titer normalized phage stocks arising from each round was performed on digoxin-ovalbumin conjugate. The percentage of

positive clones arising in rounds 3, 4 and 5 was established by monoclonal phage ELISA of 96 isolates after each round. A positive was arbitrarily defined as an absorbance greater than 0.5 with a background signal rarely above 0.01. *MvaI* fingerprinting was applied to 24 positive clones from rounds 3, 4 and 5.

### C. FACS screening

An aliquot of phagemid containing, ABLE<sup>TM</sup>C glycerol stock was scraped into 1ml of 2xTY (2% glucose, 100µgml<sup>-1</sup> ampicillin) to give an OD at 600nm of approximately 0.1cm<sup>-1</sup>. After shaking vigorously at 37°C for 2h, IPTG was added to 1mM and the culture shaken at 25°C for 4h. 50µl of culture was labeled with 100nM BODIPY<sup>TM</sup>-digoxigenin (Daugherty *et al.*, 1999) in 1ml of 5xPBS for 1h at room temperature with moderate agitation. For the last 10min of labeling, propidium iodide was added to 2µgml<sup>-1</sup>. Cells were pelleted and resuspended in 100µl of labeling mix. Scanning was performed with Becton-Dickinson FACSsort, collecting 10<sup>4</sup> events at 1500s<sup>-1</sup>.

For FACS library sorting, the cells were grown in terrific broth and induced with 0.1mMIPTG. Sorting was performed on 10<sup>6</sup> events (10<sup>7</sup> for round 2) in exclusion mode at 1000s<sup>-1</sup>. Collected sort liquor was passed through 0.7µm membrane filters and colonies allowed to grow after placing the filter on top of SOC agar plus appropriate antibiotics at 30°C for 24h.

### D. Analysis of phage clones

Screening phage particles by ELISA is summarized as follows. Binding of phage in ELISA is detected by primary sheep anti-M13 antisera (CP laboratories or 5 prime - 3 prime) followed by a horseradish peroxidase (HRP) conjugated anti-sheep antibody (Sigma). Alternatively, a HRP-anti-M13 conjugate can be used (Pharmacia). Plates can be blocked with 2% MPBS or 3% BSA-PBS. For the polyclonal phage ELISA, the technique is generally as follows: coat MicroTest III flexible assay plates (Falcon) with 100 µl per well of protein antigen. Antigen is normally coated overnight at 4°C at a concentration of 10-100 µg/ml in either PBS or 50 mM sodium hydrogen carbonate, pH 9.6. Rinse wells 3 times with PBS, by flipping over the ELISA plates to discard excess liquid, and fill well with 2% MPBS

or 3% BSA-PBS for 2 hr at 37°C. Rinse wells 3 times with PBS. Add 10 µl PEG precipitated phage from the stored aliquot of phage from the end of each round of selection (about 10<sup>10</sup> tfu.). Make up to 100 µl with 2% MPBS or 3% BSA-PBS. Incubate for 90 min at rt. Discard the test solution and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Add appropriate dilution of HRP-anti-M13 or sheep anti-M13 antisera in 2% MPBS or 3% BSA-PBS. Incubate for 90 min at rt, and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. If sheep anti-M13 antisera is used, incubate for 90 min at rt, with a suitable dilution of HRP-anti-sheep antisera in 2% MPBS or 3% BSA and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Develop with substrate solution (100 µg/ml TMB in 100 mM sodium acetate, pH 6.0, add 10 µl of 30% hydrogen peroxide per 50 ml of this solution directly before use). Add 100 µl to each well and leave at rt for 10 min. A blue color should develop. Stop the reaction by adding 50 µl 1 M sulfuric acid. The color should turn yellow. Read the OD at 450 nm and at 405 nm. Subtract OD 405 from OD 450.

Monoclonal phage ELISA can be summarized as follows. To identify monoclonal phage antibodies the pHEN phage particles need to be rescued: Inoculate individual colonies from the plates in C10 (after each round of selection) into 100 µl 2xTY containing 100 µg/ml ampicillin and 1 % glucose in 96-well plates (Corning 'Cell Wells') and grow with shaking (300 rpm.) overnight at 30°C. Use a 96-well transfer device to transfer a small inoculum (about 2 µl) from this plate to a second 96-well plate containing 200 µl of 2xTY containing 100 µg/ml ampicillin and 1% glucose per well. Grow shaking at 37°C for 1 hr. Make glycerol stocks of the original 96-well plate, by adding glycerol to a final concentration of 15%, and then storing the plates at -70°C. To each well (of the second plate) add VCS-M13 or M13KO7 helper phage to an moi of 10. Stand for 30 min at 37°C. Centrifuge at 1,800g. for 10 min, then aspirate off the supernatant. Resuspend pellet in 200 µl 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Grow shaking overnight at 30°C. Spin at 1,800 g for 10 min and use 100 µl of the supernatant in phage ELISA as detailed above.

Production of soluble antibody fragments is summarized as follows: the selected pHEN needs to be infected into HB2151 and then induced to give soluble expression of

antibody fragments for ELISA. From each selection take 10  $\mu$ l of eluted phage (about  $10^5$  t.u.) and infect 200  $\mu$ l exponentially growing HB2151 bacteria for 30 min at 37°C (waterbath). Plate 1, 10, 100  $\mu$ l, and 1:10 dilution on TYE containing 100  $\mu$ g/ml ampicillin and 1% glucose. Incubate these plates overnight at 37°C. Pick individual colonies into 100  $\mu$ l 2xTY containing 100  $\mu$ g/ml ampicillin and 1% glucose in 96-well plates (Corning 'Cell Wells'), and grow with shaking (300 rpm.) overnight at 37°C. A glycerol stock can be made of this plate, once it has been used to inoculate another plate, by adding glycerol to a final concentration of 15% and storing at -70°C. Use a 96-well transfer device to transfer a small inocula (about 2  $\mu$ l) from this plate to a second 96-well plate containing 200  $\mu$ l fresh 2xTY containing 100  $\mu$ g/ml ampicillin and 0.1% glucose per well. Grow at 37°C, shaking until the OD at 600 nm is approximately 0.9 (about 3 hr). Once the required OD is reached add 25  $\mu$ l 2xTY containing 100  $\mu$ g/ml ampicillin and 9 mM IPTG (final concentration 1 mM IPTG). Continue shaking at 30°C for a further 16 to 24 hr. Coat MicroTest III flexible assay plates (Falcon) with 100  $\mu$ l per well of protein antigen. Antigen is normally coated overnight at rt at a concentration of 10-100  $\mu$ g/ml in either PBS or 50 mM sodium hydrogen carbonate, pH 9.6. The next day rinse wells 3 times with PBS, by flipping over the ELISA plates to discard excess liquid, and block with 200  $\mu$ l per well of 3% BSA-PBS for 2 hr at 37°C. Spin the bacterial plate at 1,800 g for 10 min and add 100  $\mu$ l of the supernatant (containing the soluble scFv) to the ELISA plate for 1 hr at rt. Discard the test solution and wash three times with PBS. Add 50  $\mu$ l purified 9E10 antibody (which detects myc-tagged antibody fragments) at a concentration of 4  $\mu$ g/ml in 1 % BSA-PBS and 50  $\mu$ l of a 1:500 dilution of HRP-anti-mouse antibody in 1 % BSA-PBS. Incubate for 60 min at rt, and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Develop with substrate solution (100  $\mu$ g/ml TMB in 100 mM sodium acetate, pH 6.0. Add 10  $\mu$ l of 30% hydrogen peroxide per 50 ml of this solution directly before use). Add 100  $\mu$ l to each well and leave at rt for 10 min. A blue color should develop. Stop the reaction by adding 50  $\mu$ l 1 M sulphuric acid. The color should turn yellow. Read the OD at 450 nm and at 405 nm. Subtract OD 405 from OD 450.

Inserts in the library can be screened by PCR screening using the primers designated LMB3: CAG GAA ACA GCT ATG AC (SEQ ID NO:13) and Fd seq1: GAA TTT TCT GTA TGA GG (SEQ ID NO:14). For sequencing of the VH and VL, use is recommend of

the primers FOR\_LinkSeq: GCC ACC TCC GCC TGA ACC (SEQ ID NO:15) and pHEN-SEQ: CTA TGC GGC CCC ATT CA (SEQ ID NO:16).

## EXAMPLE 6

### Summary of Methodology for Use of the Griffin.1 Library

Methodology for using the Griffin.1 library can be summarized as follows. The Griffin.1 library is a scFv phagemid library made from synthetic V-gene segments. The library was made by recloning the heavy and light chain variable regions from the lox library vectors (Griffiths *et al.*, EMBO J, 1994) into the phagemid vector pHEN2. A kit for use of the library will contain a tube of the synthetic scFv Library (1 ml), a glycerol stock of the positive control (TG1 containing an anti-thyroglobulin clone), a glycerol stock of the negative control (TG1 containing pHEN2), a glycerol stock of *E. coli* TG1 (Gibson, 1984) suppressor strain (K12, del(lac-pro), supE, thi, hsdD5/F'traD36, proA+B+, lacIq, lacZdelM15) for propagation of phage particles (the strain supplied is a T-phage resistant variant of this), a glycerol stock of *E. coli* HB2151 (Carter *et al.*, 1985) and non-suppressor strain (K12, ara, del(lac-pro), thi/F'proA+B+, lacIq, lacZdelM15) for expression of antibody fragments. The library is kept frozen at -70°C until needed.

The strains are plated and then are grown up as overnight cultures (shaking at 37°C) of each in 2xTY containing 100 µg/ml ampicillin and 1% glucose. Cultures are diluted 1:100 with 2xTY (2xTY is 16g Typtone, 10g Yeast Extract and 5g NaCl in 1 litre) containing 100 µg/ml ampicillin and 1% glucose and the phagemids rescued by following the procedures described below. A 1:100 mixture is used of positive and the negative control together for one round of selection on immunotubes, coated with thyroglobulin.

The protocol for use of the library is summarized as follows. Phage/phagemid infect F+-*E. coli* via the sex pili. For sex pili production and efficient infection *E. coli* must be grown at 37°C and be in log phase (OD at 600 nm of 0.4-0.6). Throughout the following protocol such a culture is needed. It can be prepared as follows: transfer a bacterial colony from a minimal media plate into 5 ml of 2xTY medium and grow shaking overnight at 37°C. Next day, subculture by diluting 1:100 into fresh 2xTY medium, grow shaking at 37°C until

OD 0.4-0.6 and then infect with phage. A variety of helper phages are available for the rescue of phagemid libraries. VCS-M13 (Stratagene) and M13KO7 (Pharmacia) can be purchased in small aliquots, larger quantities for rescue of phagemid libraries can be prepared as follows: Infect 200  $\mu$ l *E. coli* TG1 (or other suitable strain) at OD 0.2 with 10  $\mu$ l serial dilutions of helper phage (in order to get well separated plaques) at 37°C (waterbath) without shaking for 30 min. Add to 3 ml molten H-top agar (42°C) and pour onto warm TYE (note 7) plates. Allow to set and then incubate overnight at 37°C. Pick a small plaque into 3-4 ml of an exponentially growing culture of TG1 (see above). Grow for about 2 hr shaking at 37°C. Inoculate into 500 ml 2xTY in a 2 litre flask and grow as before for 1 hr and then add kanamycin (25  $\mu$ g/ml in water) to a final concentration of 50-70  $\mu$ g/ml. Grow for a further 8 - 16 hr. Spin down bacteria at 10,800 g for 15 min. To the phage supernatant add 1/5 volume PEG/NaCl (20% polyethylene glycol 6000-2.5 M NaCl) and incubate for a minimum of 30 min on ice. Spin 10,800 g for 15 min. Resuspend pellet in 2 ml TE and filter sterilise the stock through a 0.45  $\mu$ m filter (Minisart NML; Sartorius). Titre the stock and then dilute to about  $1 \times 10^{12}$  p.f.u./ml. Store aliquots at -20°C. All spins are performed at 4°C, unless otherwise stated.

For growth of the library, the procedure is summarized as follows: inoculate the whole of the bacterial library stock (about  $1 \times 10^{10}$  clones) into 500 ml 2xTY containing 100  $\mu$ g/ml ampicillin and 1% glucose. Grow with shaking at 37°C until the OD at 600 nm is 0.5, this should take about 1.5-2 hours. Infect 25 ml ( $1 \times 10^{10}$  bacteria) from this culture with VCS-M13 or M13KO7 helper phage by adding helper phage in the ratio of 1:20 (number of bacterial cells:helper phage particles, taking into account that 1 OD bacteria at 600 nm = around  $8 \times 10^8$  bacteria/ml).

Spin the infected cells at 3,300 g for 10 min. Resuspend the pellet gently in 30 ml of 2xTY containing 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml kanamycin. Add 470 ml of prewarmed 2xTY containing 100  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml kanamycin and incubate shaking at 30°C overnight. The phage can be concentrated and any soluble antibodies removed (as in TG1 suppression of the amber stop codon encoded at the junction of the antibody gene and gIII is never complete) by precipitating with Polyethylene glycol (PEG) 6000. Spin the

culture from A6 at 10,800 g for 10 min (or 3,300 g for 30 min). Add 1/5 volume PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) to the supernatant. Mix well and leave for 1 hr or more at 4°C. Spin 10,800 g for 30 min. Resuspend the pellet in 40 ml water and add 8 ml PEG/NaCl. Mix and leave for 20 min or more at 4°C. Spin at 10,800 g for 10 min or 3,300 g for 30 min and then aspirate off the supernatant. Respin briefly and then aspirate off any remaining PEG/NaCl. Resuspend the pellet in 5 ml PBS and spin 11,600 g for 10 min in a microcentrifuge to remove most of the remaining bacterial debris. Store the phage supernatant at 4°C for short term storage or in PBS, 15% glycerol for longer term storage at -70°C. To titre the phage stock dilute 1 µl phage in 1 ml PBS and use 1 µl of this to infect 1 ml of TG1 at an OD600 0.4-0.6. Plate 50 µl of this, 50 µl of a 1:102 dilution and 50 µl of a 1:104 on TYE plates containing 100 µg/ml ampicillin and 1 % glucose and grow overnight at 37°C. Phage stock should be  $10^{12}$ - $10^{13}$ /ml.

Selection on immunotubes is summarized as follows. Coat Nunc-immunotube (Maxisorp Cat. No. 4-44202) overnight with 4 ml of the required antigen. The efficiency of coating can depend on the antigen concentration, the buffer and the temperature. Usually 10-100 µg/ml antigen in PBS or 50 mM sodium hydrogen carbonate, pH 9.6 at room temperature (rt), is used. Next day wash tube 3 times with PBS (simply pour PBS into the tube and then pour it immediately out again). Fill tube to brim with 2 % MPBS. Cover and incubate at 37°C (or rt according to the stability of antigen) for 2 hr to block. Wash tube 3 times with PBS. Add  $10^{12}$  to  $10^{13}$  cfu. phage, from A13, in 4 ml of 2 % MPBS. Incubate for 30 min at rt rotating continuously on an under-and-over turntable and then stand for at least a further 90 min at rt. Throw away the unbound phage in the supernatant. For the first round of selection wash tubes 10 times with PBS containing 0.1% Tween-20, then 10 times with PBS to remove the detergent. Each washing step is performed by pouring buffer in and immediately out. For the second and subsequent rounds of selection wash tubes 20 times with PBS containing 0.1% Tween-20, then 20 times with PBS. Shake out the excess PBS from the tube and elute phage by adding 1 ml 100 mM triethylamine (700 µl triethylamine (7.18 M) in 50 ml water, diluted on day of use) and rotating continuously for 10 min on an under-and-over turntable. During the incubation, tubes are prepared with 0.5 ml 1M Tris, pH 7.4 ready to add the eluted 1 ml phage, from 7, for quick neutralisation. Phage can be stored

at 4°C or used to infect TG1 as described above. After elution add another 200 µl of 1M Tris, pH 7.4 to the immunotube to neutralise the remaining phage in the tube. Take 9.25 ml of an exponentially growing culture of TG1 and add 0.75 ml of the eluted phage. Also add 4 ml of the TG1 culture to the immunotube. Incubate both cultures for 30 min at 37°C (waterbath) without shaking to allow for infection. Pool the 10 ml and 4 ml of the infected TG1 bacteria and take 100 µl to make 4-5 100-fold serial dilutions. Plate these dilutions on TYE containing 100 µg/ml ampicillin and 1% glucose. Grow overnight at 37°C. Take the remaining infected TG1 culture and spin at 3,300 g for 10 min. Resuspend the pelleted bacteria in 1 ml of 2xTY and plate on a large Nunc Bio-Assay dish (Gibco-BRL (note 8)) of TYE containing 100 µg/ml ampicillin and 1% glucose. Grow at 30°C overnight, or until colonies are visible.

For further rounds of selection, add 5-6 ml of 2xTY, 15% glycerol to the Bio-Assay dish of cells and loosen the cells with a glass spreader. After inoculating 50-100 µl of the scraped bacteria to 100 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose, store the remaining bacteria at -70°C. Once again it is a good idea to check starting OD at 600 nm is  $\leq 0.1$ . Grow the bacteria with shaking at 37°C until the OD at 600 nm is 0.5 (about 2 hr). Infect 10 ml of this culture with VCS-M13 or M13KO7 helper phage by adding helper phage in the ratio of 1:20 (number of bacterial cells:helper phage particles, taking into account that 1 OD bacteria at 600 nm = around  $8 \times 10^8$  bacteria/ml). Incubate without shaking in a 37°C water bath for 30 min. Spin the infected cells at 3,300 g for 10 min. Resuspend the pellet gently in 50 ml of 2xTY containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and incubate shaking at 30°C overnight. Take 40 ml of the overnight culture and spin at 10,800g for 10 min or 3,300 g for 30 min. Add 1/5 volume (8 ml) PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) to the supernatant. Mix well and leave for 1 hr or more at 4°C. Spin 10,800g for 10 min or 3,300 g for 30 min and then aspirate off the supernatant. Respin briefly and then aspirate off any remaining dregs of PEG/NaCl. Resuspend the pellet in 2 ml PBS and spin 11,600 g for 10 min in a micro centrifuge to remove most of the remaining bacterial debris. 1 ml of this phage can be stored at 4°C and the other 1 ml aliquot can be used for the next round of selection. Repeat the selection for another 2-3 rounds.

Screening phage particles by ELISA is summarized as follows. Binding of phage in ELISA is detected by primary sheep anti-M13 antisera (CP laboratories or 5 prime - 3 prime) followed by a horseradish peroxidase (HRP) conjugated anti-sheep antibody (Sigma). Alternatively, a HRP-anti-M13 conjugate can be used (Pharmacia). Plates can be blocked with 2% MPBS or 3% BSA-PBS. For the polyclonal phage ELISA, the technique is generally as follows: coat MicroTest III flexible assay plates (Falcon) with 100 µl per well of protein antigen. Antigen is normally coated overnight at rt at a concentration of 10-100 µg/ml in either PBS or 50 mM sodium hydrogen carbonate, pH 9.6. Rinse wells 3 times with PBS, by flipping over the ELISA plates to discard excess liquid, and block with 200 µl per well of 2% MPBS or 3% BSA-PBS for 2 hr at 37°C. Rinse wells 3 times with PBS. Add 10 µl PEG precipitated phage from the stored aliquot of phage from the end of each round of selection (about 10<sup>10</sup> cfu.). Make up to 100 µl with 2% MPBS or 3% BSA-PBS. Incubate for 90 min at rt. Discard the test solution and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Add appropriate dilution of HRP-anti-M13 or sheep anti-M13 antisera in 2% MPBS or 3% BSA-PBS. Incubate for 90 min at rt, and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. If sheep anti-M13 antisera is used, incubate for 90 min at rt, with a suitable dilution of HRP-anti-sheep antisera in 2% MPBS or 3% BSA and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Develop with substrate solution (100 µg/ml TMB in 100 mM sodium acetate, pH 6.0. Add 10 µl of 30% hydrogen peroxide per 50 ml of this solution directly before use). Add 100 µl to each well and leave at rt for 10 min. A blue colour should develop. Stop the reaction by adding 50 µl 1 M sulphuric acid. The colour should turn yellow. Read the OD at 450 nm and at 405 nm. Subtract OD 405 from OD 450.

Monoclonal phage ELISA can be summarized as follows. To identify monoclonal phage antibodies the pHEN phage particles need to be rescued: Inoculate individual colonies from the plates in C10 (after each round of selection) into 100 µl 2xTY containing 100 µg/ml ampicillin and 1 % glucose in 96-well plates (Corning 'Cell Wells') and grow with shaking (300 rpm.) overnight at 37°C. Use a 96-well transfer device to transfer a small inoculum (about 2 µl) from this plate to a second 96-well plate containing 200 µl of 2xTY containing 100 µg/ml ampicillin and 1 % glucose per well. Grow shaking at 37°C for 1 hr. Make

glycerol stocks of the original 96-well plate, by adding glycerol to a final concentration of 15%, and then storing the plates at  $-70^{\circ}\text{C}$ . To each well (of the second plate) add 25  $\mu\text{l}$  2xTY containing 100  $\mu\text{g/ml}$  ampicillin, 1 % glucose and 109 pfu VCS-M13 or M13KO7 helper phage. Stand for 30 min at  $37^{\circ}\text{C}$ , then shake for 1 hr at  $37^{\circ}\text{C}$ . Spin 1,800 g. for 10 min, then aspirate off the supernatant. Resuspend pellet in 200  $\mu\text{l}$  2xTY containing 100  $\mu\text{g/ml}$  ampicillin and 50  $\mu\text{g/ml}$  kanamycin. Grow shaking overnight at  $30^{\circ}\text{C}$ . Spin at 1,800 g for 10 min and use 100  $\mu\text{l}$  of the supernatant in phage ELISA as detailed above.

Production of soluble antibody fragments is summarized as follows: the selected pHEN needs to be infected into HB2151 and then induced to give soluble expression of antibody fragments for ELISA. From each selection take 10  $\mu\text{l}$  of eluted phage (about 105 t.u.) and infect 200  $\mu\text{l}$  exponentially growing HB2151 bacteria for 30 min at  $37^{\circ}\text{C}$  (waterbath). Plate 1, 10, 100  $\mu\text{l}$ , and 1:10 dilution on TYE containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose. Incubate these plates overnight at  $37^{\circ}\text{C}$ . Pick individual colonies into 100  $\mu\text{l}$  2xTY containing 100  $\mu\text{g/ml}$  ampicillin and 1% glucose in 96-well plates (Corning 'Cell Wells'), and grow with shaking (300 rpm.) overnight at  $37^{\circ}\text{C}$ . A glycerol stock can be made of this plate, once it has been used to inoculate another plate, by adding glycerol to a final concentration of 15% and storing at  $-70^{\circ}\text{C}$ . Use a 96-well transfer device to transfer a small inocula (about 2  $\mu\text{l}$ ) from this plate to a second 96-well plate containing 200  $\mu\text{l}$  fresh 2xTY containing 100  $\mu\text{g/ml}$  ampicillin and 0.1% glucose per well. Grow at  $37^{\circ}\text{C}$ , shaking until the OD at 600 nm is approximately 0.9 (about 3 hr). Once the required OD is reached add 25  $\mu\text{l}$  2xTY containing 100  $\mu\text{g/ml}$  ampicillin and 9 mM IPTG (final concentration 1 mM IPTG). Continue shaking at  $30^{\circ}\text{C}$  for a further 16 to 24 hr. Coat MicroTest III flexible assay plates (Falcon) with 100  $\mu\text{l}$  per well of protein antigen. Antigen is normally coated overnight at rt at a concentration of 10-100  $\mu\text{g/ml}$  in either PBS or 50 mM sodium hydrogen carbonate, pH 9.6. The next day rinse wells 3 times with PBS, by flipping over the ELISA plates to discard excess liquid, and block with 200  $\mu\text{l}$  per well of 3% BSA-PBS for 2 hr at  $37^{\circ}\text{C}$ . Spin the bacterial plate at 1,800 g for 10 min and add 100  $\mu\text{l}$  of the supernatant (containing the soluble scFv) to the ELISA plate for 1 hr at rt. Discard the test solution and wash three times with PBS. Add 50  $\mu\text{l}$  purified 9E10 antibody (which detects myc-tagged antibody fragments) at a concentration of 4  $\mu\text{g/ml}$  in 1 % BSA-PBS and 50  $\mu\text{l}$  of a 1:500 dilution of HRP-anti-mouse

antibody in 1 % BSA-PBS. Incubate for 60 min at rt, and wash three times with PBS-0.05% Tween 20, then 3 times with PBS. Develop with substrate solution (100 µg/ml TMB in 100 mM sodium acetate, pH 6.0. Add 10 µl of 30% hydrogen peroxide per 50 ml of this solution directly before use). Add 100 µl to each well and leave at rt for 10 min. A blue colour should develop. Stop the reaction by adding 50 µl 1 M sulphuric acid. The colour should turn yellow. Read the OD at 450 nm and at 405 nm. Subtract OD 405 from OD 450.

Inserts in the library can be screened by PCR screening using the primers designated LMB3: CAG GAA ACA GCT ATG AC (SEQ ID NO:13) and Fd seq1: GAA TTT TCT GTA TGA GG (SEQ ID NO:14). For sequencing of the VH and VL, use is recommend of the primers FOR\_LinkSeq: GCC ACC TCC GCC TGA ACC (SEQ ID NO:15) and pHEN-SEQ: CTA TGC GGC CCC ATT CA (SEQ ID NO:16).

#### EXAMPLE 7

##### Isolation of scFV Antibodies Specific to TNB from a Repertoire Library

This example summarizes the screening of a repertoire antibody library to the ligand TNB (trinitrobenzene). Library screening was initiated by first carrying out three rounds of phage panning of a repertoire library (Griffin -1 library) using standard protocols (see example 6, also described in <http://www.mrc-cpe.cam.ac.uk/~phage/glp.html>). Phage rescued from various rounds of panning were used to infect the *E. coli* ABLE C. The cells were grown to mid-exponential phase, induced for expression of scFv antibodies as described above and labeled with 100 nM TNBS conjugated to the fluorescent dye Cy5. The labeled cells were analyzed by flow cytometry using a Cytomation MoFlo instrument equipped with a 5 mW diode laser emitting at 633 nm. Highly fluorescent clones were isolated on membrane filters and analyzed further. Three out of 10 clones isolated by FACS were analyzed further and found to exhibit strong binding to a TNBS-BSA conjugate. Sequence analysis confirmed that one of the TNBS specific clones had also been found by phage display. However, the two other clones isolated by the present invention (periplasmic expression of the library and FACS screening) did not correspond to any of the clones isolated by phage panning.

## EXAMPLE 8

### Detection of Oligonucleotide Probes by Antibodies Expressed in the *E. coli* Periplasm

This example shows that modified oligonucleotides can diffuse through the outer membrane of bacteria. An oligonucleotide with the sequence 5'-digoxigenin-AAAAA-fluorescein-3' (designated dig-5A-FL, molecular weight of 2,384 Da, SEQ ID NO:22) containing four nuclease resistant phosphorothioate linkages between the five A residues was synthesized and purified (RP HPLC) by Integrated DNA Technologies, IA. The digoxigenin moiety of this oligonucleotide can be recognized by scFv antibodies specific to digoxin (anti-digoxin scFv). Cells expressing the anti-digoxin scFv in the periplasm may bind 5A-FL which in turn should render the cells fluorescent, provided that the probe molecule can diffuse through the outer membrane.

ABLE<sup>TM</sup>C cells expressing periplasmic scFv specific for either atrazine (Hayhurst 2000) as a negative control (FIG. 8, panels i and iii) or digoxigenin (FIG. 8, panels ii and iv) were incubated in 5x strength PBS (see example 3) together with either 100nM of digoxigenin-BODIPY<sup>TM</sup> or 100nM of dig-5A-FL. Propidium iodine was also added to serve as a viability stain. Viable cells were gated on the basis of propidium iodine exclusion (to identify cells with an intact membrane) and side scatter. Approximately 10,000 cells were analyzed at a rate of 1,000 events per second. The resulting data are shown in FIG. 8. Cells expressing an unrelated anti-atrazine antibody that does not bind to the probe exhibited only background fluorescence. In contrast, cells displaying the anti-digoxin scFv antibody became clearly labeled with both the digoxigenin-BODIPY<sup>TM</sup> as well as with 5-A-FL. The latter probe gave a signal that was clearly higher than that observed with the control cells. Even though 5-A-FL gave a lower fluorescence intensity compared to the smaller and uncharged the digoxigenin-BODIPY, the signal obtained with the former probe was sufficient for the screening of scFv libraries by FACS.

## EXAMPLE 9

### Flow Cytometric Discrimination of *E. coli* Expressing the *Fusarium solani*

#### Lipase Cutinase Using Commercial Fluorescent Substrates

This example demonstrates that commercially available fluorescent substrates can be used to specifically label *E. coli* cells displaying the relevant enzymes in their periplasm. Surprisingly, the fluorescent product of these reactions is sufficiently retained within the cell to allow for the discrimination and selection of enzyme expressing *E. coli* from non-enzyme expressing bacteria.

The gene encoding *Fusarium solani* lipase cutinase was constructed by total gene synthesis and placed downstream of the strong inducible promoter pBAD in plasmid pBAD18Cm. Protein expression from the pBAD promoter is beneficial for the screening of protein libraries by FACS (Daugherty et al. 1999). The resulting plasmid encoding the cutinase gene was designated pKG3-53-1. pKG3-53-1, and pBAD18Cm as a control, were both transformed into DH5a. In this example, the ability to discriminate cells expressing cutinase (DH5a(pKG3-53-1)) from control cells was determined using two different commercially available substrates: Fluorescein dibutyrate or LysoSensor Green DND-189 (LSG) (both from Molecular Probes, OR). The latter is a positively charged fluorescent probe that detects pH changes in the cell occurring due to ester hydrolysis by the enzyme.

Cells were grown overnight with vigorous shaking at 37°C in terrific broth/chloramphenicol 50µg/ml (TB/Cm). Subcultures were made from 100µl of overnight culture in 10ml of TB/Cm(50µg/ml). These subcultures were grown with vigorous shaking at 37°C to OD<sub>600</sub>=0.6. Four ml aliquots of the subcultures were pelleted at 3650 rpm for 20 minutes in a Beckman Allegra 6R Centrifuge. The supernatant was removed, and the pellets were resuspended in 4ml of M9 minimal media containing 0.2% glucose and chloramphenicol (Cm) at 50µg/ml. Arabinose, from a 20% stock, was added to a final concentration of 0.2%. The cultures were induced at 25°C with vigorous shaking for 4 hours. Subsequently, 2 ml aliquots of the induced cultures were pelleted at 8000rpm for 10 minutes in an Eppendorf 5415C Centrifuge, washed with fresh media and pelleted again at 8000rpm for 10 min. The washed pellets were resuspended in M9 salts media without

glucose to an optical density  $OD_{600}=1.0$ . The stock solution was diluted 1:10 and 1 ml of the diluted cell suspension was mixed with 0.1 ml 0.1 mM Fluorescein dibutyrate (FDB) stock solution in dimethyl sulfoxide (DMSO). The final FDB concentration was 10 $\mu$ M. Reactions were allowed to proceed at 37°C for 30 minutes. The labeled cells were immediately analyzed on a Becton Dickinson FACSsort equipped with an Ar 488nm laser. The fluorescence distribution of the cutinase expressing cells and the control cells is shown in FIG. 9A.

The utility of a second probe for the discrimination between positive (enzyme expressing) and control cells was also examined. *E. coli* expressing cutinase from the pKG3-53-4 plasmid, and negative cells (expressing the unmodified pBAD18Cm plasmid) were grown, induced and washed as above. The pellet was washed with 1% sucrose, pelleted again, and resuspended in fresh 1% sucrose to  $OD_{600}=1.0$ . This stock solution of cells was kept on ice.

For labeling, a LysoSensor Green DND-189 (LSG, Molecular Probes) stock solution was prepared to 1mM in DMSO. Also, a 1 M 4-Nitrophenyl Butyrate stock solution was prepared in DMSO. Cell labeling was initiated by first diluting the cell stock solution, adding the LSG to a final concentration of 1  $\mu$ M and diluting the 4-Nitrophenyl Butyrate 1:1000 to give a final concentration of 1  $\mu$ M. The enzymatic hydrolysis of 4-Nitrophenyl Butyrate by the cells was allowed to proceed at 25°C for 5 minutes and the cells were then immediately analyzed on a Becton Dickinson FACSsort as above. The fluorescence distribution of the cutinase expressing cells and the control cells stained with the LysoSensor Green DND-189 probe is shown in Figure 9B.

\* \* \*

All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the

steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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